Investigating the above-ground application of EPNs for the control of the vine mealybug *Planococcus ficus*

by

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DECLARATION

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ABSTRACT

The table and wine grape industries in South Africa are of major economic importance, particularly within the Western Cape Province, making the pest control of grapevines a priority. The vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) is a key pest of South African grapevines, damaging vines by phloem feeding, by disfiguring grapes with waxy residues, by encouraging the growth of sooty moulds, and by serving as a vector for viruses. Chemical insecticides like chlorpyrifos have traditionally been used in their control, though the cryptic habitats on the vine chosen by the most economically significant mealybug life stage complicates pesticide application. Additionally, mealybugs excrete a waxy coating that repels liquids, and their short generation time allows the rapid development of resistance to chemical pesticides.

Consequently, alternatives are sought for the control of mealybugs on grapevines. One candidate for their control is entomopathogenic nematodes (EPNs), which are nematode parasites of soil-based insect life stages. Of major interest in this respect are the EPNs of the families Steinernematidae and Heterorhabditidae, the infective juveniles (IJs) of which have been successfully applied to control soil-based insect pests. However, the maladaptation of IJs to non-soil environments (such as foliage) has limited their use as biocontrol agents above ground, due to their susceptibility to extremes of temperature and to prolonged exposure to ultraviolet light (UV), as well as their generally low tolerance for desiccation. The aim of this study was to investigate EPN candidates for the control of *P. ficus*, and to develop methods for overcoming the weaknesses of EPNs in foliar application.

As new species of EPNs are constantly being described, laboratory-based bioassays were performed, screening three newly described EPN species (*Steinernema jeffreyense*, *Heterorhabditis noenieputensis*, and *Steinernema* spp. WS9), as well as *Steinernema yirgalemense*, for their control of *P. ficus*. *Heterorhabditis noenieputensis* was the most effective, causing 90% ± 3% mortality, followed by *S. yirgalemense* (63% ± 7%), with both mortalities being significantly greater than was

that of the control. The presence of the nematodes within the body cavities of *P. ficus* cadavers was confirmed. *Steinernema yirgalemense* was selected as the EPN candidate of choice for experiments going forward, due to the difficulty in mass-producing *H. noenieputensis*. However, developments in the formulation methods of the *Heterorhabditid* species will warrant the re-examination of *H. noenieputensis* in future.

On performing a laboratory bioassay to determine the minimum amount of time required for the optimal infectivity of *P. ficus* by *S. yirgalemense*, the mortality of *P. ficus* was found not to improve significantly for individuals exposed to *S. yirgalemense* for longer than 3h. Subsequently, the effects of varying temperature and relative humidity (%RH) on the ability of *S. yirgalemense* to cause mortality in *P. ficus* were tested. The mortality of *P. ficus* was greatest at 25°C (72% ± 3%), and at 100% RH, during the humidity trial. Each result established targets for the optimal application of *S. yirgalemense*.

The ability of two adjuvants, Zeba® and Nu-Film-P®, to improve the efficacy of *S. yirgalemense* applications was tested under semi-controlled conditions. The combination of Zeba® and Nu-Film-P® in suspension with *S. yirgalemense* was shown to deposit significantly more EPNs (30.8 ± 4 IJs / 4 cm^2) onto grapevine leaves in the laboratory than did formulations with EPNs and water alone, or with EPNs and Nu-Film-P®, though not significantly more than with EPNs and Zeba® alone. A growth chamber bioassay was conducted to assess the effect of the addition of the adjuvants to *S. yirgalemense* suspensions on *P. ficus* mortality. The addition of Zeba® and Nu-Film-P® to *S. yirgalemense* caused significantly higher mortality (84% ± 5%) in *P. ficus* in the growth chamber than did any other treatment, including EPNs + Zeba® (47% ± 3%), after 48h. A bioassay carried out in the greenhouse showed similar results, with the *S. yirgalemense* treatment containing Zeba® and Nu-Film-P® causing 88% ± 3% mortality after 48h, which was significantly higher than was that which was attained with any other EPN treatment.

The treatments were then assessed under semi-field conditions that would be capable of inflicting the harshest environmental stress. Application of *S. yirgalemense* (at a concentration of

4000 IJs/ml) + Zeba® + Nu-Film-P® to *P. ficus* individuals on grapevine leaf discs hung on grapevines resulted in 66% ± 4% P. *ficus* mortality after 48h, which was significantly higher ($p < 0.01$) than was achieved using either *S. yirgalemense* + Zeba® alone, or EPNs + water alone, though overall less than the control obtained in the glasshouse. A bioassay to assess the impact of reducing EPN concentration was performed, resulting in predictable reductions in *P. ficus* mortality when progressively lower concentrations of *S. yirgalemense* (3000, 2000 and 1000 IJs/ml) were applied with Zeba® and Nu-Film-P® to *P. ficus* on grapevine leaf discs. The control obtained by the formulation containing 3000 IJs/ml was significantly greater than was that which was achieved with each other treatment after 48h (44% ± 4%), though the control overall was lower than was attained with the 4000 IJs/ml concentration used in the previous bioassay. This demonstrates that the EPN concentration remains important to the efficacy of EPN applications.

So as to assess the effects of climatic conditions on EPN longevity, a time-of-day application bioassay was performed. *Steinernema yirgalemense* was formulated with Zeba® and Nu-Film-P® and applied directly to grapevines, the leaves of which were removed and rinsed at timed intervals, whereupon the live nematodes present on them were counted. The experiment was carried out at 8:00 (with conditions being 14.6°C and 93.2% RH at application), and repeated at 14:00 (with conditions being 31.0°C and 39.9% RH at application). Higher numbers of living nematodes were recorded on the grapevine leaves at all of the time intervals concerned during the 8:00 trial when compared with the same intervals during the 14:00 trial, indicating that the higher percentage RH had a greater effect on IJ survival than did the more optimal temperature (but lower % RH) during the afternoon trial.

This study represents an additional step towards the successful utilization of EPNs (in this case, *S. yirgalemense*) as biocontrol agents of *P. ficus* on grapevines in South Africa. *Steinernema yirgalemense* can achieve > 66 % mortality of *P. ficus* under semi-field conditions, when the humidity (which is the critical factor for IJ survival on foliage) is effectively managed. Future work should examine *S. yirgalemense* in full-field application, as well as available methods (such as the use of

irrigation, or shade netting) for maximizing the relative humidity immediately following IJ application.

OPSOMMING

Die tafel- en wyndruif industrie is van groot ekonomiese belang in Suid-Afrika, veral in die Wes-Kaap provinsie. Die beheer van wingerd peste is daarom uiters belangerik. Die wingerd witluis, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), is een van die belangrikste peste in Suid-Afrikaanse wingerde en veroorsaak skade deur te voed op die floëem van die plant, deur die druiwetrosse te besmet met wasagtige afskeidings, deur swart swamgroei aan te moedig en dien ook as ‘n draer van virusse. Chemiese insekdoders soos chlorpirifos word tradisioneel gebruik vir die beheer van die wingerd witluis. Die aanwending van sulke insekdoders word egter bemoeilik deur benutting van kriptiese lewenswyse op die wingerd van die mees skadelike witluis lewensfase. Boonop skei wingerd witluise ‘n waslagie af wat vloeistowwe afweer en hul kort generasie tyd stel hul in staat om weerstand te ontwikkel tot chemiese plaagdoders.

Daarom word daar alternatiewe metodes vir die beheer van wingerd witluise ondersoek. Entomopatogeniese nematodes (EPNs) is parasiete van grondlewende lewensfases van insekte en een van die kandidate vir die beheer van wingerd witluis. Van groot belang in hierdie nematodes is die EPNs van die families Steinernematidae en Heterorhabditidae, waarvan die infektiewe larwes (IJs) al suksesvol aangewend is om grondlewende insek peste te beheer. IJs is egter nie aangepas om bo grondvlak te oorleef nie, aangesien hul sensitief is vir uiterste temperature en langdurige blootstelling van UV strale, asook ’n lae toleransie het vir uitdroging. Dit beperk die gebruik van IJs as biologiese beheermiddels in omgewings bo grondvlak, soos op die blare van die wingerd. Die doel van hierdie studie was om EPN kandidate te identifiseer vir die beheer van *P. ficus*, en metodes te ontwikkel om die probleme van EPNs in die aanwending op blare te oorkom.

Omdat nuwe spesies van EPNs voortdurend beskryf word, was drie nuut beskryfde species is gebruik vir biotoetse in die laboratorium (*Steinernema jeffreyense*, *Heterorhabditis noenieputensis*, *Steinernema* spp. WS9), asook *Steinernema yirgalemense*. Hul vermoë om *P. ficus* te beheer was ondersoek. Resultate toon dat *Heterorhabditis noenieputensis* die mees effektief was met 90% ± 3%

mortaliteit, gevolg deur *S. yirgalemense* (63% ± 7%), albei se mortaliteit was beduidend groter as die van die kontrole. Die aanwesigheid van nematodes in die liggaamsholtes van *P. ficus* kadawers was bevestig. *Steinernema yirgalemense* was gekies as die EPN kandidaat vir toekomstige eksperimente, en mootlike probleme met die massaproduksie van *H. noenieputensis*. Alhoewel, toekomstige ontwikkeling in die massatelings metodes van *Heterorhabditid* spesies sal beteken dat *H. noenieputensis* heroorweeg sal kan word as 'n belowende biobeheer agent.

Met biotoetse in die laboratorium om te bepaal wat is die minimum tydperk vir *S. yirgalemense* om *P. ficus* optimaal te infekteer, was daar gevind dat die mortaliteit nie beduidend verbeter het na 3 h van blootstelling aan *S. yirgalemense* nie. Gevolglik was die effek van verskillende temperature en relatiewe humiditeit (%RH) op die vermoë van *S. yirgalemense* om mortaliteit in *P. ficus* te veroorsaak, getoets. Die mortaliteit van *P. ficus* was die hoogste by 25°C (72% ± 3%), en by 100% RH, gedurende die humiditeit toets. Elke resultaat het mikpunte gelewer vir die optimale aanwending van *S. yirgalemense*.

Die vermoë van twee byvoegingsmiddels, Zeba® en Nu-Film-P®, om die doeltreffendheid van *S. yirgalemense* aanwendings te verhoog, was getoets onder semi-beheerde toestande. Die kombinasie van Zeba® en Nu-Film-P® in suspensie met *S. yirgalemense* het beduidend meer EPNs (30.8 ± 4 IJs / 4 cm^2) op die wingerdblare in die laboratorium tot gevolg gehad as die suspensies met slegs EPNs, slegs water of met EPNs en Nu-Film-P®, alhoewel nie beduidend meer as die suspensies met slegs EPNs en Zeba® nie. 'n Groeikamer biotoets was uitgevoer om die effek van die byvoeging van byvoegingsmiddels tot die *S. yirgalemense* suspensies op *P. ficus* mortaliteit te bepaal. Die byvoeging van Zeba® en Nu-Film-P® tot *S. yirgalemense* het beduidend hoër mortaliteit (84% ± 5%) in *P. ficus* in die groeikamer veroorsaak as enige ander behandeling, insluitend EPNs + Zeba® (47% ± 3%), na 48 h. 'n Biotoets wat uitgevoer was in die glashuis het soortgelyke resultate gelewer, met die behandeling wat Zeba® en Nu-Film-P® bevat, wat 88% ± 3% mortaliteit veroorsaak het na 48 h. Dit was beduidend hoër as met enige ander EPN behandeling.

Die toediening van *S. yirgalemense* was toe getoets onder semi-veld toestande, wat in staat sou wees om die ongunstige omgewingstoestande te veroorsaak. Aanwending van *S. yirgalemense* (teen 'n konsentrasie van 4000 IJs/ml) + Zeba® + Nu-Film-P® tot *P. ficus* individuele op wingerdblaar skyfies wat gehang is op wingerde, het gelei tot 66% ± 4% insek mortaliteit na 48 h, wat beduidend hoër was as die resultate van die aanwending van slegs *S. yirgalemense* + Zeba® of slegs EPNs en water, alhoewel minder as vir die kontrole in die glashuis. 'n Biotoets was ook uitgevoer om die impak van 'n laer EPN konsentrasie te bepaal. Soos verwag, was *P. ficus* mortaliteit verlaag met verminderde konsentrasies van *S. yirgalemense* (3000, 2000 en 1000 IJs/ml) aangewend is met Zeba® en Nu-Film-P® op *P. ficus* op wingerdblaar skyfies. Die mortaliteit in die kontrole van die suspensies van 3000 IJs/ml was beduidend meer as die van enige ander behandeling na 48 h (44% ± 4%), alhoewel die kontrole laer was as die mortaliteit wat bereik was met 'n 4000 IJs/ml konsentrasie wat gebruik was in die vorige biotoets. Die resultate toon dat die konsentrasie van EPNs belangerik bly in die doeltreffendheid van EPN aanwendings.

Om die effek van klimaatstoestande op EPN oorlewing is getoets gebaseer was op die tyd gedurende dit dag wanneer die biotoets uitgevoer is. *Steinernema yirgalemense* was geformuleer met Zeba® en Nu-Film-P® en direk aangewend op wingerdblare. Die blare was dan verwyder en afgespoel op sekere intervalle en die nematodes aanwesig op die blare getel. Die eksperiment was uitgevoer om 8:00 (met toestande van 14.6°C en 93.2% RH by aanwending), en herhaal om 14:00 (met toestande van 31.0°C en 39.9% RH by aanwending). Hoër getalle lewende nematodes was waargeneem op die wingerdblare by alle intervalle van die 8:00 proewe in vergelyking met dieselfde intervalle by die 14:00 proewe, wat aandui dat die hoër persentasie RH 'n groter effek gehad het op die oorlewing van die nematodes as die meer optimale temperatuur (maar laer % RH) van die middag proef.

Die studie bied In addisionele stap nader aan die suksesvolle gebruik van EPNs (in hierdie geval, *S. yirgalemense*) as biologiese beheermiddel van *P. ficus* op wingerde in Suid-Afrika. *Steinernema yirgalemense* kan > 66 % mortaliteit van *P. ficus* tot gevolg hê onder semi-veld

toestande, wanneer die humiditeit (wat die kritiese faktor is vir die oorlewing van IJs op blare) effektief bestuur word. In toekomstige navorsing moet die aanwending van *S. yirgalemense* in volle veldkondisies ondersoek, asook beskikbare metodes (soos die gebruik van besproeiing of skadunette) vir die maksimalisering van relatiewe humiditeit direk nadat IJs aangewend is.

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CHAPTER 1

Entomopathogenic Nematodes to Control Above-Ground Insect Pests, with Potential Use Against the Vine Mealybug, Planococcus ficus: A review

ABSTRACT

The vine mealybug *Planococcus ficus* (Hemiptera: Pseudococcidae) is a major pest of grapevines in South Africa. The efficacy of chemical pesticides against *P. ficus* is limited by the development of resistance. The most economically important life stage of *P. ficus* forms colonies in cryptic refuges on the vine and in the grape bunches. Entomopathogenic nematodes (EPNs) are soil-based insect-parasitic roundworms of the families Heterorhabditidae and Steinernematidae, which are successfully used as biological control agents of soil-based insect pests in many countries, especially Europe and the USA. The potential of these nematodes as biological control agents has led to research into their use in the control of above-ground pests. Laboratory based studies showed exceptionally good control in most cases, as the life stages of above-ground insect pests have not co-evolved with EPNs and thus are more susceptible than subterranean life stages. However, limitations such as the need for moisture and UV sensitivity makes above-ground application of EPNs problematic. This paper gives an up-to-date overview of research into the application of EPNs as a biocontrol agent for the control of insect pests in a foliar, or above-ground, context.

Key Words: entomopathogenic nematodes, Heterorhabditidae, integrated pest management, mealybug, *Planococcus ficus*, Steinernematidae, foliar application

INTRODUCTION

Entomopathogenic nematodes (EPNs) are soil-based roundworms in the order Rhabditida, characterised by their exclusive pathogenicity to insects via mutualism with symbiotic bacteria (Griffin *et al.*, 2005). Various nematode families have been investigated as potential biocontrol agents, with over 30 having been linked to insects in some way (Kaya & Stock, 1997). These include Mermithidae, Tetradonematidae, Allantonematidae, Phaenopsitylenchidae, Sphaerulariidae, Steinernematidae and Heterorhabditidae (Lacey *et al.*, 2001). Current research focuses almost entirely on Steinernematidae and Heterorhabditidae (Grewal *et al.*, 2005). Nematodes of other families have proven to be mostly unsuitable as commercial biocontrol agents, due to a variety of factors. These include habitat sensitivity, intolerance to chemicals, or a lack of cost-effective methods of mass production, all of which have limited research into these families (Lacey *et al.*, 2001).

Mealybugs (Hemiptera: Pseudococcidae) are scale insects characterised by a white, waxy ("mealy") secretion that covers the bodies of nymphs and adult females (Downie & Gullan, 2004). The presence of this secretion is characteristic of the family, being present on all individuals with the exception of *Dysmicoccus*, which possesses reduced waxy secretions, and *Misericoccus*, which has none at all (McKenzie, 1967). All mealybugs are phytophagous, possessing piercing-sucking mouthparts that allow them to access the phloem to feed (Millar, 2002). Mealybugs are important pests of South African grapevines, causing damage by their feeding, the secretion of honeydew, which encourages growth of sooty moulds, and by serving as vectors of plant diseases (Millar, 2002). Mealybugs feed on all parts of the vine (Godfrey *et al.*, 2002) and the vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), is the predominant mealybug pest of South African vineyards (Walton *et al.*, 2004). South Africa is the second largest producer of wine and table grapes in the southern hemisphere (after Chile), with wine production reaching 1 044 million litres in 2007 (FAO, 2009), and table grape production at 59.4 million 4.5 kg-equivalent cartons during the period 2014-2015 (SATI, 2015).

Investigations have been conducted into the possible above-ground application of EPNs, ever since interest was first shown in their use as biocontrol agents. In the current review, an up-to-date overview is given of the progress that has been made in the use of EPNs applied above-ground to control of insect pests and the potential of using EPNs to control mealybugs on grapevines.

Entomopathogenic nematodes

Life cycle

EPNs belonging to the families Steinernematidae and Heterorhabditidae have been applied with great success as a biocide against a wide range of pest insects (Campos-Herrera, 2015). These two families have similar traits and life cycles, despite them not being closely related (Blaxter *et al.*, 1998). Characteristic of EPNs is their entomophagy by means of symbiosis with an enteric bacterium. *Steinernema* is associated with bacteria of the genus *Xenorhabdus*, whereas *Heterorhabditis* is associated with *Photorhabdus* (Griffin *et al.*, 2005). Steinernematids and heterorhabditids have a free-living stage, the infective juvenile (IJ), which is also known as the dauer juvenile. This stage occurs free in the soil, where they actively locate a suitable insect host. This is also the stage that will be cultured and used in the above-ground applications.

Occurrence and distribution

In South Africa, the first record of an EPN was made in relation to the black maize beetle, *Heteronychus arator* Fabricius (Coleoptera: Scarabaeoidea), which was collected from a maize field near Grahamstown in the Eastern Cape province (Harington, 1953). EPNs were first applied to above-ground insect life stages in South Africa in the 1980s against the larval stages of the sugarcane borer, *Eldana saccharina* Walker (Spaull, 1992).

An investigation into the biological control of the banded fruit weevil, *Phlyctinus callosus* (Schönerr) (Coleoptera: Curculionidae), from 1993 to 1994, yielded a heterorhabditid that was later confirmed to be *Heterorhabditis bacteriophora* Poinar (Grenier *et al.*, 1996a, b). Since the description of the first new EPN species for South Africa in 2006 as *Steinernema khoisanae* Nguyen, Malan and

Gozel (Nguyen *et al.*, 2006), several other descriptions of new species and records of occurrence have followed. To date, a total of 16 EPN species have been reported from South Africa, of which five are heterorhabditids, and 11 are steinernematids. Three of the five species of heterorhabditids and 10 of the 11 species of steinernematids were new species (Malan *et al.*, 2016).

Use in biological control

EPNs have been successfully commercialised for use against insect pests in North America, Europe, Japan, China and Australia (Ehlers, 1996; Kaya *et al.*, 2006), with research in other countries still being in the relatively preliminary stages (Kaya *et al.*, 2006). The most widely used commercial applications of EPNs for insect control have been aimed at the soil-based stages of insect life cycles (Wilson & Gaugler, 2004). Above-ground application against foliage feeding insects has been rare, with it generally having been less successful than soil-based application (Shapiro-Ilan *et al.*, 2006).

Arthurs *et al.* (2004) conducted a metastudy of 136 trials concerning the above-ground application of *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding (Nematoda: Steinernematidae), which has, to date, been the most commonly used species for control of above-ground insect pests. The study showed that EPN efficacy varied according to targeted habitat. The most favourable habitat was boreholes (the tunnels made by boring insects into foliage, fruit and trunks), followed by cryptic habitats (habitats protected from exposed conditions by foliage or other conditions), with exposed habitats being the least successful. EPN efficacy also varied by trial location – laboratory application (the most controlled environment) was generally most successful, followed by greenhouse, with field application being the least successful.

Most studies on the above-ground application of EPN to control insects have targeted the order Lepidoptera, while other studies have also targeted Coleoptera, Diptera, Hemiptera, Hymenoptera and Thysanoptera (Table 1). The above-ground stages of insects have been targeted with nematodes in different environments, including laboratory conditions, covered areas such as shade houses and

glasshouses, and, large-scale field applications, whereas the micro habitat of the insect itself can be boring, cryptic or exposed (Table 2).

Coleoptera

As major pest insects, the true weevil family (Coleoptera: Curculionidae) has been a focus for biological control via EPNs. *Steinernema feltiae* Filipjev (Nematoda: Steinernematidae) has been investigated for the control of *Scolytus* (Fabricius) (Coleoptera: Curculionidae), where it has been found to be ineffective in controlling the overwintering populations of the curculionid larvae at the doses applied (Finney & Walker, 1979). On applying a variety of EPN species to *Stethobaris nemesis* (Prena & O'Brien, 2011) (Coleoptera: Curculionidae) that were kept on leaf discs in the laboratory, Shapiro-Ilan & Mizell (2012) found that *S. feltiae* and *S. carpocapsae* both exhibited high levels of *S. nemesis* mortality.

Coleopteran pests that have been targeted with foliar application of EPNs include the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), which is a pest of potato foliage. The adult weevil has been targeted with *S. carpocapsae*, resulting in infection rates of 30-60% when applied to foliage in an agar solution (MacVean *et al.*, 1982). The addition of agar to the nematode suspension, increased viability and infectivity, resulting in a significant reduction in the amount of leaf damage that is caused by *L. decemlineata* (Adel & Hussein, 2010; Hussein *et al.*, 2012).

In South Africa, the banded fruit weevil (*Phlyctinus callosus* Schönerr) (Coleoptera: Curculionidae) tends to emerge above ground during the late spring and early summer (Myburgh *et al.*, 1973) in vineyards and orchards, where it is a serious pest. Ferreira and Malan (2014) assessed the pathogenicity of indigenous *Heterorhabditis zealandica* (Poinar) (Rhabditida: Heterorhabditidae) and *Heterorhabditis bacteriophora* Poinar (Nematoda: Heterorhabditidae) to adults of the banded fruit weevil in the laboratory. Application of high concentrations of 400 IJs/insect, and an exposure

time of 4 days, resulted in mortality of 41-73% on banded fruit weevil larvae, and 13-45% on adults, under optimum conditions.

Diptera

Many Dipteran species (particularly of the family Agromyzidae) are leaf-miners and present a challenge to farmers, as chemical control methods are limited on edible leafy crops for reasons of human health. In this respect, biological control methods such as EPNs represent an attractive alternative.

Harris *et al.* (1990) showed that applications of *S. carpocapsae* achieved mortality levels of 64% on larvae of the American serpentine leaf miner, *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), on chrysanthemum, which was equivalent to the effect obtained with applications of the insecticide and antihelminthic abamectin. Further investigation by LeBeck (1993) determined that all larval instars of *L. trifolii* were susceptible to the depredations of *S. carpocapsae*, with the second instar being the most susceptible. Investigations into the susceptibility of *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) to EPNs determined that all instars of *L. huidobrensis* larvae were susceptible to *S. feltiae* (Williams & Walters, 1994, 2000), with the second larval instar being found to be the most susceptible at relatively low humidity (Williams & Macdonald, 1995). The aforementioned research was consolidated by Williams and Walters (2000), who applied *S. feltiae* to Chinese cabbage plants infested with *L. huidobrensis*. This resulted in *L. huidobrensis* mortality of 82%, which was a significant increase over the results that were achieved with use of heptenophos, a chemical control method. Investigations concerning *L. trifolii* primarily found that abamectin was more effective than was *S. carpocapsae*, when the former was applied to lima beans (Hara *et al.*, 1993) and chrysanthemums (Broadbent & Olthof, 1995).

The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and the Natal fruit fly *Ceratitis rosa* (Karsch) (Diptera: Tephritidae) were tested for vulnerability to EPNs, with the adult stages (i.e. the above-ground stages) of both being shown to be susceptible to infection by EPNs. However, the

stages concerned were found to be less susceptible than soil-based larvae, making soil-based EPN applications probably more feasible (Malan & Manrakan, 2009).

Hemiptera

Investigations into the use of *S. feltiae* for control of the silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) found that, while *S. feltiae* was unable to achieve significant control of *B. tabaci* by itself (inducing pest mortality of between 10-32% on tomato, cucumber, verbena, poinsettia, and chrysanthemum), the effect of nematode application could be enhanced by 15-31% with the use of adjuvants (Head *et al.*, 2004). Combining applications of *S. feltiae* with imidacloprid provided significantly more comprehensive control than did the use of either treatment alone (Cuthbertson *et al.*, 2007). Five species of EPNs were tested to determine their biocontrol potential against the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae), a hemipteran pest of ornamental plants. It was found that there was potential for *C. ciliata* to be controlled with EPNs, particularly *Heterorhabditis indica* Poinar, Karunakar & David (Shapiro-Ilan & Mizell, 2012).

Mealybugs (family Pseudococcidae) are among the most important pests in South African agriculture, and work is ongoing to develop methods of foliar application of EPNs against them. *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) is the main pest of citrus, while *P. ficus* is the major pest of grapevines, and the obscure mealybug *Pseudococcus viburni* (Signoret) (Hemiptera: Pseudococcidae), is regarded as the main mealybug pest of deciduous fruit (Prinsloo & Uys, 2015)

The citrus mealybug is capable of infesting high percentages of citrus trees, including the fruit (Hattingh & Moore, 2003).Van Niekerk and Malan (2012) screened potential EPN candidates for the foliar control of *P. citri*, finding *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler and Adams and *H. zealandica* to be the most effective nematode species. They then tested both species in combination with various agrochemicals and natural enemies, and neither species was shown to decrease in infectivity. Both EPN species were however highly infective to the larvae of the

mealybug ladybird *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae) (Mulsant), which is a biocontrol predator of *P. citri*, indicating that these organisms should not be used together as part of an IPM system (Van Niekerk & Malan, 2014a).

Van Niekerk and Malan (2015) then investigated the use of adjuvants to overcome a key obstacle to the application of EPNs to foliage, namely maintaining levels of relative humidity (RH) to allow for EPN infection of the citrus mealybug. Application of the adjuvant Zeba® increased the effectiveness of *H. zealandica* against *P. citri* by 22% at 80% RH and a combination of both Zeba® and Nu-Film-P® significantly increased the amount of nematode deposition on leaves. In a semi-field trial in a citrus orchard, significantly higher control was achieved by adding Zeba® with a resulting 53% control. The study showed that the addition of an adjuvant improved the ability of *S. yirgalemense* to infect *P. citri* by retarding desiccation and buffering the nematodes from the harsh environmental conditions (Van Niekerk & Malan, 2014b).

Stokwe and Malan (2016) investigated the ability of EPNs to control *P. viburni*, one of three species of pseudococcids that are commonly found on pome fruit in the Western Cape Province of South Africa (Wakgari & Giliomee, 2004). They found that *H. zealandica* and *S. yirgalemense* were both able to reproduce in *P. viburni*, with *H. zealandica* displaying greater mealybug penetration, and also possessing the ability to infect *P. viburni* at the centre of infested apple cores, making it a potential candidate for foliar control of *P. viburni* in apple and pear orchards.

Hymenoptera

To date, most research into the application of EPNs for the control of hymenopteran pests of foliage has focused on sawflies. Georgis and Hague (1988) evaluated *S. feltiae* for use against the web-spinning larch sawfly *Cephalcia lariciphila* (Wachtl) (Hymenoptera: Pamphiliidae) in Welsh larch. They found infection of larval stages to be prohibitively low, compared to application at equivalent rates, to prepupae in the soil (3-39% versus 61% infection, respectively).

Vincent and Bélair (1992) took a similar approach, applying *S. carpocapsae* to dwarf apple trees, in efforts to control the apple sawfly, *Holocampa testudinea* (Klug) (Hymenoptera: Tenthredinidae). Though the application of EPNs in such a case was found not to impact a significant amount of primary damage to the fruit, in terms of leaving of scars as a result of burrowing. However, it did significantly reduce the amount of secondary damage incurred, in terms of the number of frass pellets deposited at the entry point of burrowing. Further research by Bélair and Vincent (1992) assessed the application of *S. carpocapsae* against *H. testudinea* over 3 years. Primary damage to apple fruit by *H. testudinea* was reduced by 98% and 100% in the first 2 years, while the percentage of fruits exhibiting secondary damage was significantly reduced after a single application of *S. carpocapsae*. The effectiveness of the treatment was attributed to the cages used, which increased the RH, and therefore nematode longevity and mobility.

Lepidoptera

Research by Bélair *et al.* (1999) into the application of *S. carpocapsae* against the oblique banded leafroller, *Choristoneura roseceana* (Harris) (family Tortricidae), a pest of apples, concluded that the low efficacy of the nematode and the inability of the selected adjuvants to improve nematode efficacy, indicate that the use of *S. carpocapsae* as a sole agent against the leafroller could not be recommended. Kaya and Reardon (1982) assessed the efficacy of *S. carpocapsae* in controlling the Western spruce budworm *Choristoneura occidentalis* (Walsingham) (family Tortricidae) in fir, and concluded that significant infectivity of Western spruce budworm larvae and pupae could not be obtained, even when adjuvants were used and treated branches were bagged in an effort to enhance nematode survivability,.

Cydia pomonella (Linnaeus) (Lepidoptera: Tortricidae), the codling moth, has been a major target of research into the foliar application of EPNs, due to its status as a serious pest of apples worldwide. The application of *S. feltiae* to codling moth diapausing larvae in corrugated cardboard on apple tree trunks resulted in 80% codling moth mortality in mid-autumn, with 32% mortality in midsummer (Kaya *et al.*, 1981). Unruh and Lacey (2001) assessed the effect of application of a variety

of methods on the infectivity of *S. carpocapsae* to codling moth larvae trapped in cardboard traps in apple orchards in Washington, USA, finding that the application of EPNs to traps containing codling moth larvae was most effective in the relatively cool and humid conditions in the morning and evening, as well as in the case of both the pre- and the post-wetting of treatments. Odendaal *et al.* (2015) performed an investigation into South African EPNs and their ability to control codling moth in South African environments, assessing local species *Steinernema jeffreyense* Malan, Knoetze & Tiedt (Nematoda: Steinernematidae) and *S. yirgalemense* against commercially available nematodes *S. feltiae*, and two strains of *H. bacteriophora*. They found that *S. jeffreyense* showed highest efficacy (67%) when it was applied to codling moth larvae that were kept in small mesh cages. No adjuvants were added in the above-mentioned trial, with the cages being sprayed with water every 2 hours for the first 6 hours of the trial. The above-mentioned study indicates the potential for South African nematodes to be effective under South African conditions, if high humidity can be maintained.

Codling moth infestations have been shown to be persistent due to the contamination of fruit bins in orchards, even when other control methods were in place. Lacey *et al.* (2005) examined the ability of *S. carpocapsae* and *S. feltiae* in controlling the infestation of orchard fruit bins, finding that both species provided high mortality of cocooned codling moth larvae when they were applied together with wetting agents, as well as when they were applied by immersing fruit bins in nematode suspensions.

Two studies have been conducted in South Africa to determine the potential of using EPNs for the control of codling moth infesting wooden fruit bins. De Waal *et al.* (2010) used 25 IJs/ml as a discriminating dosage in laboratory trials and determined the LD90 of codling moth to be 100 IJs/ml using miniature bins under optimum conditions.. The study also indicated that high humidity is crucial to obtaining the desired control and covering it with a tarpaulin, together with the use of adjuvants were found to improve the control significantly. Three EPNs, including a local isolate, *S. yirgalemense*, and two commercial isolates, *S. feltiae* and *H. bacteriophora*, were evaluated for their potential to control codling moth in miniature bins at a concentration of 25 IJs/ml (Odendaal *et al.*,

2016 a & b). The best control of codling moth was obtained using *S. feltiae* (75%), with the degree of control being significantly increased to >95% by the addition of adjuvants.

Stem-boring lepidopteran larvae are attractive candidates for EPN application, as they burrow holes into stems and leaves, which are protected from harsh environmental conditions. Chief among such larvae are the sesiids (Lepidoptera: Sesiidae), mostly obligate borers of plant stems. Kaya and Brown (1986) investigated the ability of *S. feltiae* to control the large red-belted clearwing, *Synanthedon culciformis* (Linnaeus) (Lepidoptera: Sesiidae) on alder, and the sycamore borer *Synanthedon resplendens* (Edwards) (Lepidoptera: Sesiidae) on sycamore. The researchers found *S. feltiae* to be more effective against *S. culciformis* larvae when it was applied directly to borer galleries, due to the *S. culciformis* residing in the alder heartwood, which is moister than sycamore heartwood and thus retards IJ desiccation. Deseo and Miller (1985) performed similar experiments, applying *S. feltiae* to apple trees in Italy to control two strains of red-belted clearwing *Synanthedon myopaeformis* (syn. *S. typhiaeformis*) (Borkhausen) (Lepidoptera: Sesiidae). They concluded that the two specific strains of *S. feltiae* were capable of actively seeking out and migrating towards *S. myopaeformis*.

More recently, the effects of EPNs against sesiids on peach have been assessed. Cossentine *et al.* (1990) applied *H. bacteriophora* (heliothidis strain) to control the peach tree borer, *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae), finding that a suspension of EPNs in and around the boreholes failed to result in a significantly reduced number of adults emerging from the holes. Cottrell *et al.* (2011), in testing several EPN species for efficacy against the lesser peachtree borer *Synanthedon pictipes* (Grote & Robinson) (Lepidoptera: Sesiidae), compared the action of an adjuvant (polyacrylamide gel) with the application of moistened diapers to treated areas, with the aim of improving the moisture retention and UV protection qualities. It was found that both techniques improved the control of *S. pictipes* compared to the control.

Shannag & Capinera (1995) assessed *S. carpocapsae* for the control of melonworm, *Diaphania hyalinata* (Linnaeus) (Lepidoptera: Crambidae) on squash foliage. Field applications resulted in

infection rates of up to 55%, though the survival of nematodes on foliage was only 0.25% after 18 hours in moderate humidity conditions,

Shapiro-Ilan *et al.* (2010) applied *S. carpocapsae* for control of late instars of the lesser peach tree borer, *S. pictipes*, using a post-application covering of latex paint, moistened infant's nappy, or gel spray, so as to enhance the nematode survival rate on the peach tree foliage. Application of Barricade® gel post nematode application was effective in enhancing the efficacy of *S. carpocapsae* against peach tree borers on the foliage. Further research established that Barricade® could be used in a single spray with *S. carpocapsae*, and that the combination was at least as successful as was chlorpyrifos, which is the accepted chemical standard for use against the lesser peach tree borer (Shapiro-Ilan *et al.*, 2016).

The different life stages of the South American tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), have been tested using EPNs with a view to foliar application. Van Damme *et al.* (2016) showed in laboratory studies that all insect instars were susceptible to infection by *S. feltiae*, *H. bacteriophora* and *S. carpocapsae*, with *S. feltiae* causing 100% mortality under optimum laboratory conditions. They found that improvements to spraying conditions and the addition of adjuvants allowed IJ concentrations as low as 6.8 IJs/cm^2 to achieve levels of control equivalent to the recommended IJ concentration of 27.3 IJs/cm^2 under standard conditions.

Thysanoptera

The major thysanopteran pest targeted with EPNs is the western flower thrip, *Frankliniella occidentalis* (Pergande) (family Thripidae), due to its preference for residing in cryptic habitats on plants. Buitenhuis and Shipp (2005) also assessed the efficacy of *S. feltiae* against *F. occidentalis* by using wetting agents and by applying nematodes to flowering stage chrysanthemums versus the vegetative stage (i.e. exposed), but found no significant difference in the amount of mortality that was caused by the application of either stage, and in addition, observing no significant mortality caused by *S. feltiae* in the case of adult thrips. Arthurs and Heinz (2006) assessed applications of *S.*

feltiae against thrips on chrysanthemums, but failed to reduce the amount of damage caused to the host plant.

Challenges to above-ground application

Unlike chemical pesticides, EPNs are living creatures and consequently their success as biocontrol agents is dependent on their survival. This makes EPN application less user-friendly and higher-maintenance than chemical control methods. Environmental factors that limit EPN survival above ground include temperature, ultraviolet light (UV) light and moisture/relative humidity (%RH).

Temperature

Nematodes are highly susceptible to changes in temperature and must therefore be kept in aqueous solutions of 4-30°C, with most species being intolerant to temperatures higher than 35°C for longer than 30 min at a time (Grewal *et al.*, 1994). Higher temperatures also reduce the solubility of oxygen in solution. Depriving EPNs of oxygen for prolonged periods of time results in their deactivation and ultimate death (Wright *et al.*, 2005). Different EPN species also have different thermal niches within which they can infect and establish within their respective hosts. Grewal *et al.* (1994) list the temperature niches for various species of nematodes in their interactions with last-instar *Galleria mellonella* Linnaeus (Tortricidae: Pyralidae) larvae. In order to minimise the negative effects of temperature, nematodes should be applied only at optimum temperatures in a glasshouse and field application should take place either in early morning or late afternoon. Nematodes such as *S. feltiae*, which are tolerant to low temperatures, can be selected for use in cooler environments.

Ultraviolet (UV) light

Exposure to UV light should be taken into consideration when applying EPNs above ground. UV light and sunlight have been shown to significantly affect the behaviour and pathogenicity of both plant- (Godfrey & Hoshino, 1933) and animal-parasitic (Stowens, 1942) nematodes. Gaugler and Boush (1978) observed the effects of short UV radiation and natural sunlight on *S. carpocapsae*, in terms of their interactions with *G. mellonella* larvae. They found that the irradiation of IJs caused

reduced pathogenicity and increased larval survival time post-infection after 7 min of exposure to short-term UV radiation, while exposure to direct sunlight also reduced pathogenicity by as much as 95% after 60 min. Gaugler *et al.* (1992) found that *S. carpocapsae* IJs were rendered completely inactive after 10 minutes of moderate UV exposure, whereas *H. bacteriophora* was significantly affected after only 4 minutes, indicating that the susceptibility to UV light varies across species. In general, it is known that nematodes would move away towards cryptic micro habitats away from direct sunlight. The problem of UV light could also be avoided with the application of nematodes early in the morning or late afternoon, to give them time to move towards the cryptic micro habitat in which the target host most probably will also reside.

Humidity

Temperature and UV radiation are contributing factors to the desiccation of IJs when the latter are applied above ground. Nematode survival and viability on foliage appear to be directly related to the prevailing relative humidity (RH). Glazer (1992), comparing the survivability of *S. carpocapsae* on bean foliage at 45, 60 and 80% RH, showed that nematode survival and pathogenicity both improved at %RH, and with the addition of antidesiccants. Glazer *et al.* (1992 a & b) assessed the survival of *S. carpocapsae* IJs at low RH that were used to control the cotton pests *Earias insulana* (Boisduval) (Lepidoptera: Nolidae), *Heliothis armigera* (Hübner) (Lepidoptera: Noctuidae), and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). The addition of anti-desiccants to nematode solutions applied to cotton plants was found to result in 85-95% insect mortality, compared to 22% in the control, as well as significantly decreasing the amount of foliage damage that was incurred compared to the control.

Adjuvants

From previous research it can be concluded that one of the possible means of overcoming environmental limitations with regard to humidity in applying EPNs above-ground, is the addition of adjuvants to modify the characteristics of the nematode suspension. Adjuvants are roughly defined

as additives to pesticide solutions that are intended to increase, or to modify, their effects (Krogh *et al.*, 2003). The United States Environmental Protection Agency (EPA` 2015), in contrast, includes safeners and synergists in its definition of adjuvants. In South Africa, as the Department of Agriculture, Forestry and Fisheries (2015) guidelines regarding adjuvants are still under development, reference is therefore made to the definitions of both the co-formulant and the adjuvant in the EU regulation that collectively refers to both as "adjuvants".

Determining the toxicity of any adjuvant on the nematodes themselves is also important. Shapiro-Ilan *et al.* (2010), tested five adjuvants (Anti-Stress 2000®, Moisturin®, Nu-Film-17®, Shatter-Proof®, and Transfilm®) for their toxicity to *S. carpocapsae*, and showed that nematode survival only decreased significantly compared to the control at 40% concentration. This was high above the recommended application concentration of Shatter-Proof®, the adjuvant selected for field trials due to it being the adjuvant which yielded the lowest numerical mortality for nematodes in suspension.

Research is still being conducted into the ability of EPNs to control mealybug species, with some advances already being made in this direction. Stokwe & Malan (2016) showed evidence of the ability of *H. zealandica* to infest, and to cause mortality among, *P. viburni* on the surface of Starking apples, which could be improved with the addition of adjuvants. De Waal *et al.* (2013) determined that the addition of Zeba® to nematode solutions that were applied to tree bark for the control of diapausing codling moth, *C. pomonella* (Linnaeus) (Lepidoptera: Tortricidae), intensified the degree of humidity that was experienced in the micro-environment of the moth larvae's habitat inside the tree bark. In so doing, it served to increase nematode movement and efficacy.

Van Niekerk and Malan (2014b) assessed the efficacy of *S. yirgalemense* against *P. citri* in a citrus grove in South Africa, applying EPNs via handheld sprayer to adult female *P. citri* individuals on citrus leaf discs that were suspended from the trees. The treatments included use of the adjuvants, Nu-Film-P® and Zeba®, as well as a combination of both. The combination of Nu-Film-P® and

Zeba® achieved the highest mealybug mortality (53%), though not significantly higher than when applied with Zeba® alone (50%).

Adjuvant efficacy varies on a case-to-case basis. In testing several adjuvants in combination with EPNs for the control of the diamondback moth, *Plutella xylostella* Linnaeus, Baur *et al.* (1997) found that, while the adjuvants tested served to increase the pathogenicity of the nematodes, overall the benefit attained was probably insufficient to warrant the use of EPNs against the pest. They also observed that several of the adjuvants tested were phytotoxic to radish leaves, highlighting the importance of screening adjuvants not only for efficacy and nematode mortality, but also for host plant toxicity.

Planococcus ficus on grapevine

The vine mealybug is the dominant species of mealybug that is found in South African vineyards (Walton, 2003). *Planococcus ficus* possesses biological traits which give it an advantage over other, similar mealybug species. The combination of a high female reproductive rate and the rapid development of nymphs results in four to seven generations per year (Daane *et al*., 2008). Vine mealybugs are also not obligate pests of grapevines, sustaining populations on a wide range of hosts, including common weeds that help to sustain populations around the vineyard area (Daane *et al*., 2008).

The vine mealybug has been found to transmit grapevine leafroll virus, whose infection characteristically involves the rolling of leaves and the discolouration of limbs, reducing yield (Bovey *et al*., 1980). Mealybugs are also sap-feeders, causing reduced yield on grapes, while table grape producers also object to the disfiguring waxy residue and honeydew (causing growth of sooty mould) that mealybugs leave on grapes, rendering them unmarketable in an industry which values pristine fruit. (Geiger & Daane, 2001).

The exceptionally high susceptibility of *P. ficus* to EPNs and their tendency to form colonies in cryptic habitats above ground, made them ideal candidates for control using nematodes (Le Vieux & Malan 2013a). Applications can be to the leaves and grapevine bunches during the summer, when

the leaves form a dense canopy. Such EPN application can be done before or even during harvesting, as no problems with chemical residues to the fruit, humans or the environment will be experienced. Nematodes can also be applied only to the stem after leaf drop, as mealybug colonies are hiding in the bark. In both application scenarios the nematodes will come into contact with the soil by dripping from the leaves and stems to the soil where it can target those mealybug colonies in the soil close to the stem and on the roots.

Current control strategies

Chemical control

Pesticides remain the dominant method of pest control on plant crops. However, as public awareness of the potential dangers of chemical control has grown, which include contamination of groundwater, potential harm to humans and animals, and the development of resistance among target pests, non-chemical alternatives are continuously being investigated (Hussaini, 2002).

Pesticide application can prove problematic to populations of natural enemies. Walton and Pringle (1999) tested the effects of five pesticides against a mealybug parasitoid *Coccidoxenoides perminutus* Girault (Hymenoptera: Encyrtidae). They found that, of the five insecticides tested, chlorpyrifos, endosulfan and cypermethrin, were highly toxic to the parasitoid. Mgocheki and Addison (2009) tested the effects of five different pesticides against *Anagyrus* spp. and *C perminutus*, both two endoparasitoids of the vine mealybug. They found that α-cypermethrin and fipronil were highly toxic to the two parasitoid species involved, and that while buprofezin had no direct impact on parasitoid mortality, it did delay the emergence of adults from mealybug cadavers.

South African chemical control methods have focused mostly on the use of chlorpyrifos (Walton & Pringle, 2004) and imidacloprid (Le Vieux, 2013), with candidates such as Scorpion® (dinotefuran) and Movento® (spirotetramat) proving to be promising control agents of *P. ficus* more recently (Jones & Nita, 2016). It has been noted that application of chemical insecticides is complicated both by the waxy filaments that *P. ficus* produces, as well as by its choice of cryptic

habitats under the raised bark of the grapevine, both of which shield mealybugs from contact with chemical sprays (Berlinger, 1977). The findings made in this regard have led to the investigation of biological alternatives, or supplements, to chemical control.

Biological control

Several species have been touted as possible biological control agents of *P. ficus* in South Africa, including *Cryptolaemus montrouzieri* Mulsant (Greathead *et al.,* 1971), *Anagyrus* spp. (Hymenoptera: Encyrtidae) (Walton & Pringle, 2004), and *C. perminutus* (Walton, 2003). However, barriers exist to the use of parasitoids as biocontrol agents. Daane *et al*. (2008) performed a survey of parasitism of the vine mealybug in California vineyards, concluding that parasitism of mealybugs was low overall, due to their cryptic choice of habitat and the interference of the ant species that tended the mealybugs.

DISCUSSION

Above-ground insects such as mealybugs are expected to be susceptible to EPNs, because EPNs present a novel predator threat to the mealybugs. Additionally, the high susceptibility of *P. ficus* to EPNs under optimal conditions (i.e. those of ideal temperature and humidity) (Le Vieux, 2013) would seem to indicate the potential of EPNs as a control agent for mealybugs. EPNs are intensively used under cover, such as in glasshouses and shade houses, in which more optimal conditions prevail. Additionally, EPNs have potential value as a non-toxic alternative to manufactured chemical pesticides, thus allowing producers an additional biological tool with which to access the organic produce market.

However, field applications of EPNs against above ground pests have historically been disappointing. Foliage-based pests that reside in cryptic habitats above ground, such as beneath bark, in bore holes, or under leaves that are out of the reach of the sun, would appear to be ideal targets for EPNs that require conditions of shade, moderate temperatures, and high humidity in order to survive and to be infective. Application of EPNs to insect pests in controlled environments (such as the

laboratory, and the glasshouse) is evidence of their potential as the biocontrol agents of pests in environments in which the levels of humidity remain high, in which desiccation is relatively slow, and in which nematodes are able to use moisture post-application to find and infect insect hosts. In contrast, EPNs tend to fare poorly against pests of foliage in the field, due to their rapid desiccation rate in environments where humidity cannot be directly controlled.

In a South African study, Le Vieux and Malan (2013a, b; 2015) investigated the potential of EPNs as a biological control agent against the vine mealybug. As previous studies had indicated that the mealybugs could also occur on grapevine roots, their study mainly focused on the soil application of EPNs. In laboratory studies, the high susceptibility of the adult vine mealybug against six indigenous EPN species was shown, with the most promising being *S. yirgalemense* (Le Vieux & Malan, 2013b). In olfactometry tests, it was indicated that *S. yirgalemense* actively move towards the vine mealybug, which would be advantageous in the case of the above-ground application of the nematodes to find mealybugs fast in cryptic habitats before drying out (Le Vieux & Malan, 2015). Research against other types of mealybug have been encouraging – Van Niekerk and Malan (2012, 2014a, b, 2015) were able to demonstrate high lethality of a range of indigenous EPNs to the citrus mealybug as well as their compatibility with a variety of agrochemicals, and were able to achieve up to 53% control of citrus mealybugs on citrus trees by using a polymer adjuvant Zeba.

A variety of methods are currently being developed to counteract the desiccation challenges confronting the foliar application of EPNs. Novel application methods have been developed to retard the desiccation rates involved, from the post-application spraying of a gel that was originally used in firefighting, to the envelopment of treated areas with moistened diapers. Simple management practices such as the time of application by applying either late in the evening or early morning can play an important role in nematode efficacy, as nematodes need only a few hours of optimum conditions to be able to infect the host. We can conclude that the main barrier to successful application of EPNs in the control of foliar pests is the environment, and successful use of EPNs on foliage requires cultural and chemical methodology put in place in order to maximise the persistence and

infectivity of EPNs on foliage – be it through time-sensitive application, spray methods, adjuvant formulation, or any combination of the three.

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TABLES

Table 1.1. Insect pests whose above-ground life stages have been targeted with entomopathogenic nematodes.

Order/Scientific name	Common name	Family	Target crop	Location	References
Coleoptera:					
Leptinotarsa decemlineata	Colorado potato beetle	Chrysomelidae	Potato	Colorado, USA; Havlíčkův Brod, Czech Republic	Welch & Briand (1961); MacVean *et al*. (1982); Adel & Hussein (2010); Hussein *et al.* (2012)
Phlyctinus callosus	Banded fruit weevil	Curculionidae	Deciduous fruit; grapevine	Western Cape, South Africa	Ferreira & Malan (2014)
Scolytus scolytus	Larger European elm bark beetle	Curculionidae	Elm	Surrey, UK	Finney & Walker (1979)
Stethobaris nemesis	N/a	Curculionidae	Sycamore	Georgia, USA	Shapiro-Ilan & Mizell (2012)
Diptera:					
Ceratitis capitata	Mediterranean fruit fly	Tephritidae	Fruits	Western Cape, South Africa	Malan & Manrakan (2009)
C. rosa	Natal fly	Tephritidae	Fruits	Western Cape, South Africa	Malan & Manrakan (2009)
Liriomyza huidobrensis	Serpentine leaf miner	Agromyzidae	Leafy vegetables	York, UK; Harpenden, UK	Williams & Walters (2000); Williams & Macdonald (1995);
L. trifolii	American serpentine leaf miner	Agromyzidae	Chrysanthemum	Hawaii, USA; Ontario, Canada; California, USA	Harris *et al.* (1990); Hara *et al.* (1993); Broadbent & Olthof (1995)
Hemiptera:					
Bemisia tabaci	Silverleaf whitefly	Aleyrodidae	Cucumber; poinsettia; chrysanthemum; verbena	York, UK	Head *et al*. (2004); Cuthbertson *et al.* (2007, 2008)
Corythucha ciliata	Sycamore lace bug	Tingidae	Sycamore	Florida, USA	Shapiro-Ilan & Mizell (2012)
Planococcus citri	Citrus mealybug	Pseudococcidae	Citrus	Western Cape, South Africa	Van Niekerk & Malan (2013)
Pseudococcus viburni	Obscure mealybug	Pseudococcidae	Apple	Western Cape, South Africa	Stokwe & Malan (2015)

Order/Scientific name	Common name	Family	Target crop	Location	References
Hymenoptera:					
Cephalcia lariciphila	Hymenopteran sawflies	Pamphilidae	Larch	Wales, UK	Georgis & Hague (1988)
Hoplocampa testudinea	Apple sawfly	Tenthredinidae	Apple	Quebec, Canada	Vincent & Bélair (1992)
Lepidoptera:					
Choristoneura occidentalis	Spruce budworm	Tortricidae	Fir	Canada	Kaya *et al*. (1981); Kaya & Reardon (1982)
C. rosaceana	Oblique banded leafroller	Tortricidae	Apple	Quebec, Canada	Bélair *et al*. (1999)
Cydia pomonella	Codling moth	Tortricidae	Apple	Western Cape, South Africa	Kaya *et al.* (1984); Unruh & Lacey (2001); Odendaal *et al*. (2015); Lacey *et al*. (2005)
Diaphania hyalinata	Melonworm moth	Crambidae	Squash	Florida, USA	Shannag & Capinera (1995)
Earias insulana	Egyptian stemborer	Nolidae	Cotton	Bet Dagan, Israel	Glazer (1992)
Eldana saccharina	Sugarcane stalk borer	Pyralidae	Sugar cane	South Africa	Spaull (1992)
Euzophera semifuneralis	American plum borer	Pyralidae	Plum	New York State, USA	Kain & Agnello (1999)
Helicoverpa zea	Corn earworm	Noctuidae	Corn	Mississippi, USA	Bong & Sikorowski (1983)
Heliothis armigera	Cotton bollworm	Noctuidae	Bean	Bet Dagan, Israel	Glazer & Navon (1990)
H. viriscens	Tobacco budworm	Noctuidae	Tobacco	North Carolina, USA	Chamberlin & Dutkey (1958)
Herpetogramma phaeopteralis	Tropical sod webworm	Crambidae	Turfgrass	Florida, USA	Tofangsazi *et al*. (2014)
Hyphantria cunea	Fall webworm	Arctiidae	Cherry	Tokyo, Japan	Yamanaka *et al*. (1986)
Mamestra brassicae	Cabbage moth	Noctuidae	Cauliflower	Rumbeke-Beltem, Belgium	Beck *et al*. (2014)
Manduca sexta	Tobacco hornworm	Sphingidae	Tobacco	North Carolina, USA	Chamberlin & Dutkey (1958)
Operophtera brumata	Winter moth	Geometridae	Apple	N/a	Jacques (1967)
Ostrinia nubilalis	European corn borer	Crambidae	Cabbage	Bet Dagan, Israel	Ben-Yakir *et al*. (1998)
Phyllocnistis citrella	Citrus leaf miner	Gracillariidae	Citrus	Sydney, Australia	Beattie *et al*. (1995)
Platyptilia carduidactyla	Artichoke plume moth	Pterophoridae	Artichoke	N/a	Bari & Kaya (1984)
Plutella xylostella	Diamondback moth	Plutellidae	Kale; cabbage	Nairobi, Kenya; New Delhi, India	Nyasani *et al.* (2008); Baur *et al.* (1995, 1997, 1998); Schroer *et al.* (2005);

Order/Scientific name	Common name	Family	Target crop	Location	References
					Mason *et al.* (1998); Somvanshi *et al*, (2006)
Prionoxystus robinae	Carpenterworm	Cossidae	Oak	Kentucky, USA	Forschler & Nordin (1988)
Pryeria sinica	Euronymus leaf notcher	Zygaenidae	Japanese spindle	Korea	Lee *et al*. (2006)
Spodoptera exigua	Beet armyworm	Noctuidae	Nursery ornamentals	N/a	Begley (1990)
S. littoralis	African cotton leafworm	Noctuidae	Cotton	Bet Dagan, Israel	Glazer *et al*. (1992a, 1992b)
Synanthedon culciformis	Large red-belted clearwing	Sesiidae	Alder, Sycamore	California, USA	Kaya & Brown (1986)
S. exitiosa	Peachtree borer	Sesiidae	Apple	Italy	Deseo & Miller (1985)
S. myopaeformis	Red-belted clearwing	Sesiidae	Peach	British Columbia, Canada	Cossentine *et al*. (1990)
S. pictipes	Lesser peachtree borer	Sesiidae	Peach	Columbus, Ohio	Cottrell *et al*. (2011)
S. resplendens	Sycamore borer	Sesiidae	Alder, Sycamore	California, USA	Kaya & Brown (1986)
S. tipuliformis	Current clearwing	Sesiidae	Blackcurrant	Derwent Valley, Tasmania	Miller & Bedding (1982)
Tuta absoluta	Tomato leaf miner	Gelechiidae	Tomato	Barcelona, Spain	Batalla-Carrera *et al.,* (2010); Van Damme *et al*. 2016
Zeiraphera canadensis	Spruce bud moth	Tortricidae	Spruce	Canada	Eidt & Dunphy (1991);
Thysanoptera:					
Frankliniella occidentalis	Western flower thrip	Thripidae	Chrysanthemum, Saintpaulia	England, UK; Ontario, Canada	Buitenhuis & Shipp (2005); Arthurs & Heinz (2006)

Table 1.2. Above-ground life stages of insect pests targeted with entomopathogenic nematodes in different environments.

Family	Scientific name/family	Target crop	Insect stage	Pest habitat	Lab-oratory	Glass-house	Field	Nematode species
Agromyzidae	*Liriomyza huidobrensis*	Leafy vegetables	Larvae	Cryptic	x	x		Sf
	L. trifolii	Chrysanthemum	Larvae	Cryptic	x	x		Sc
Aleyrodidae	*Bemisia tabaci*	Cucumber; poinsettia;	Nymph; adult	Exposed	x	x		Sf; Sc
Arctiidae	*Hyphantria cunea*	Cherry	Larvae	Exposed		x	x	Sf
Chrysomelidae	*Leptinotarsa decemlineata*	Potato	Larvae	Exposed	x	x	x	Sc
Cossidae	*Prionoxystus robinae*	Oak	Larvae	Boring		x		Sf
Crambidae	*Ostrinia nubilalis*	Cabbage	Eggs, neonates	Boring	x	x	x	Sc; Hb
	Diaphania hyalinata	Squash	Larvae, prepupae,	Exposed	x			Sc; Hb; Sf; Sg
	Herpetogramma Phaeopteralis	Turfgrass	Larvae	Exposed	x	x		Sc, Sf, Hb, Hm, Hi
Curculionidae	*Phlyctinus callosus*	Apples; pears; grapevine	Larvae, Adults	Exposed	x			Hb; Hz
	Scolytus scolytus	Elm	Larvae	Boring			x	Sf
	Stethobaris nemesis	Sycamore	Nymph	Exposed	x			Sf; Sc, Hb, Hi
Gelechiidae	*Tuta absoluta*	Tomato	Larvae	Boring	x	x		Sc; Sf; Hb
Geometridae	*Operophtera brumata*	Apple	Larvae	Exposed	x			Sc
Gracillariidae	*Phyllocnistis citrella*	Citrus	Larvae	Boring			x	Sc
Noctuidae	*Helicoverpa zea*	Corn	Larvae	Boring			x	Sc
	Heliothis armigera	Bean	Larvae	Exposed	x	x		Sf
	H. viriscens	Tobacco		Exposed	x		x	Sc
	Mamestra brassicae	Cauliflower	Larvae	Exposed	x		x	Sc
	Spodoptera exigua	Nursery ornamentals	Larvae	Exposed			x	Various
	S. littoralis	Cotton	Larvae	Exposed	x			Sc, Sg, Hb
	Earias insulana	Cotton	Larvae	Exposed	x	x		Sc
Pamphilidae	*Cephalcia lariciphila*	Larch	Larvae, prepupae	Exposed			x	Sf
Plutellidae	*Plutella xylostella*	Kale; cabbage	Larvae	Exposed	x	x	x	Various)
Pseudococcidae	*Planococcus citri*	Citrus	Nymph, adult	Cryptic	x	x	x	Sy; Hz
	Pseudococcus viburni	Apple	Nymph, adult	Cryptic	x		x	Hz, Hb, Sc, Hb, Sy
Pterophoridae	*Platyptilia carduidactyla*	Artichoke	Larvae	Boring			x	Sc
Pyralidae	*Eldana saccharina*	Sugar cane	Larvae	Boring			x	H spp.

Family	Scientific name/family	Target crop	Insect stage	Pest habitat	Lab-oratory	Glass-house	Field	Nematode species
	Euzophera semifuneralis	Plum	Larvae	Boring			x	Sf, Hb
Sesiidae	*Synanthedon culciformis*	Alder; Sycamore	Larvae	Boring			x	Sf, Sb
	Synanthedon exitiosa	Apple	Larvae	Boring			x	Sf
	Synanthedon myopaeformis	Peach	Larvae	Boring			x	Hh
	Synanthedon pictipes	Peach	Larvae	Boring	x		x	Sc, Sr, Sr, Sg, Hf, H. meg, H. mex, Hb, Hz
	S. resplendens	Alder; Sycamore	Larvae	Boring			x	Sf, Sb
	S. tipuliformis	Blackcurrant	Larvae	Boring			x	Sb
Sphingidae	*Manduca sexta*	Tobacco	Larvae	Exposed	x		x	Sc
Tenthredinidae	*Hoplocampa testudinea*	Apple	Larvae	Exposed	x		x	Sf; Hb, Sf
Thripidae	*Frankliniella occidentalis*	Chrysanthemum Saintpaulia	Nymph	Cryptic		x		Sf; Hb; Tn;
Tingidae	*Corythucha ciliata*	Sycamore	Adult	Exposed	x			Hi, Hb, Hg, Sr
Tortricidae	*Choristoneura occidentalis*	Fir	Larvae	Cryptic			x	Sc
	C. rosaceana	Apple	Larvae	Cryptic	x		x	Sr; Sf; Sc; Sg
	Cydia pomonella	Apple	Larvae	Cryptic			x	Sf; Sc
	Zeiraphera canadensis	Spruce	Larvae	Cryptic			x	Sc
Zygaenidae	*Pryeria sinica*	Japanese spindle	Larvae	Exposed	x		x	Sc

CHAPTER 2

The Potential for use of Entomopathogenic Nematodes in the control of the Vine Mealybug, *Planococcus ficus*

ABSTRACT

Planococcus ficus Signoret (Hemiptera: Pseudococcidae), the vine mealybug, is the dominant mealybug pest of grapes in South Africa. Chemical control methods are limited in efficacy against mealybugs, due to their cryptic nature, waxy coating, and fast generation time, leading to the development of insecticide resistance. Entomopathogenic nematodes (EPNs) were investigated as an alternative control agent. Four EPN species were screened for efficacy against adult female *P. ficus*, the most potent of which were *Heterorhabditis noenieputensis*, with 90% mortality after 48 h, and *Steinernema yirgalemense* with 63% mortality after the same time period. Significantly, fewer nematodes of *Steinernema* spp. WS9 were found in the body cavities of dissected *P. ficus* post-application, compared to the other EPN species. *Steinernema yirgalemense* was selected over *H. noenieputensis* for further testing, as research into the mass-production of *S. yirgalemense* is ongoing. The effects of temperature and humidity on the infectivity of *S. yirgalemense* to adult female *P. ficus* were also assessed. Application of *S. yirgalemense* at 25°C yielded the highest mortality of 72%, followed by its application at 30°C resulting in 45% mortality, with its application at 15°C registering the lowest (9%) mortality. Humidity screening indicated that *S. yirgalemense* operates best at 100% relative humidity (RH) with 70% mortality of mealybugs, with lower RH levels giving correspondingly lower mortality rates (61% mortality at 85% RH, 40% mortality at 75% RH). As soil-based organisms, *S. yirgalemense* are effective as biocontrol agents of *P. ficus* under conditions of moderate temperature and high humidity. Their lethality to *P. ficus*, and their status as an indigenous species, make them highly valuable as potential biocontrol agents.

Key words: *Planococcus ficus*, vine mealybug, *Steinernema yirgalemense*, *Heterorhabditis noenieputensis*, *Steinernema jeffreyense,* entomopathogenic nematodes, laboratory, deposition, bioassay, screening

INTRODUCTION

South Africa is the twelfth largest producer of grapes in the world, producing over 1.9 million tonnes of grapes, comprising 2.61% of overall world grape production in 2014 (FAO, 2016). The wine industry in South Africa alone had an impact on the gross domestic product (GDP) of R36 billion, comprising 1.2% of total GDP for 2013 (SAWIS, 2015), while the gross value of production for table grapes in South Africa has doubled to R4 billion, from the 2006/07 season to 2013/14 (DAFF, 2015). In particular, the Western Cape Province contributed 54% of the total value of the South African wine grape industry in 2013 (SAWIS, 2015), as well as 60% of overall table grape production in 2016 (SATGI, 2016), making the Western Cape the largest grape producer in South Africa. The grape industry is, therefore, of significant economic importance to South Africa, and particularly the Western Cape.

The vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) is a key pest of grapevine, and one of 20 species of economically important members of the family Pseudococcidae in South Africa (Annecke & Moran, 1982). Surveys by Walton (2003) established *P. ficus* as the dominant mealybug species in South African vineyards, with high reproduction rates and rapidly developing nymphs, giving it an advantage over other, similar mealybug species (Daane *et al*., 2006). The vine mealybug causes damage to grapevines directly via phloem feeding, which restricts nutrient flow to fruits, thereby reducing grape yield, as well as by producing large amounts of honeydew, which encrust the leaves and stems, promoting sooty mould growth and bunch rot (Daane *et al.,* 2008). Mealybugs such as *P. ficus* also serve as vectors for the transmission of viral diseases such as grapevine leafroll-associated virus 3 (GLRaV-3) (Cabaleiro & Segura, 1997). Transmission of GLRaV-3 results in grapevines having increased sensitivity to fungal attack and environmental

change, with harvests of infected vines showing a decline in quality, especially in terms of wine production (Cabaleiro *et al.*, 2013).

Chemical control strategies for *P. ficus* are currently based around the applications of such pesticides as chlorpyrifos, prothiophos, and imidacloprid (Walton & Pringle, 2004), though mealybug physiology and habitat choices tend to complicate spray application. In addition to residing in cryptic habitats under the raised bark on the trunk of the grapevine, mealybugs produce the waxy ('mealy') secretion that gives them their name, both of which provide protection from the spray application of pesticides (Berlinger, 1977; Walton & Pringle, 2004).

Entomopathogenic nematodes (EPNs) are roundworms of the order Rhabditida that prey exclusively on insects, and consequently have been investigated as candidates for biological control agents in terms of insect control. EPNs of the families Steinernematidae and Heterorhabditidae, in particular, have been used as biocides against soil-based pests with great success (Campos-Herrera, 2015). Despite their genetic dissimilarity, Heterorhabditid and Steinernematid families share many biological traits (Blaxter *et al.*, 1998). EPNs of both families prey on insects during their infective juvenile (IJ) stage, which is nonfeeding, exists in the soil, and is the only life stage to exist outside of an insect host. IJs encounter their host, either by means of actively seeking for insects in the soil, or by means of ambushing them. Upon discovering a potential host, IJs enter the body cavity of the insect through natural openings, such as the mouth, anus and spiracles, thereupon making their way to the haemocoel (Adams & Nguyen, 2002). Some Heterorhabditid species may also use a dorsal tooth to penetrate the cuticle, whereupon they enter the haemocoel directly (Forst & Clarke, 2002).

Once inside, the IJ releases stored symbiotic bacteria from the intestine in order to kill the insect host. The bacteria belong to the genus *Xenorhabdus* in the case of *Steinernema* IJs, and to the genus *Photorhabdus* in the case of *Heterorhabditis*. The bacterium multiplies within the host, converting the internal tissues of the insect into bacterial biomass, which induces septicaemia and death in the host, usually within a period of 1 to 2 days (Adams & Nguyen, 2002). IJs feed on the bacteria, moulting successively until they reach their adult stage. In the case of steinernematids, IJs will

eventually become male and female adults, while in the case of heterorhabditids, the IJs become hermaphroditic females in the first generation, with additional amphimictic males and females in subsequent generations. The adults mate and lay eggs that hatch into first-stage juveniles, which and continue the life cycle. This continues until the food within the cadaver is depleted, at which stage second-stage juveniles store a small amount of bacteria in their digestive system, before moulting into the pre-infective and IJ stages, whereupon they leave the cadaver in search of a new host (Wright & Perry, 2002).

EPNs have proven attractive as potential biocontrol agents of insects, being successfully commercialised for such use on four different continents (Ehlers, 1996; Kaya *et al.*, 2006). Application has tended to focus on the soil-based stages in the life cycles of insect pests, as EPNs are adapted to soil environments (Wilson & Gaugler, 2004), though success has also been observed in the use of EPNs against boring insect species (Ehlers, 1996). Research into the application of EPNs for the control of foliage-based pest insect life cycle stages has been rarer and less successful than has soil application (Shapiro-Ilan *et al.*, 2006; Platt *et al*., 2017).

Pesticides remain the dominant pest control method of *P. ficus*, though concern with regards to the contamination of food chains, harm to non-target creatures (including natural enemies of the pest), and the development of resistance has prompted interest in non-chemical control methods (Hussaini, 2002).

Following the success of EPN treatments against soil-based insect pests, interest has been ignited in the use of EPNs against such insect pests of foliage as *P. ficus*. Where chemical control agents rely on direct application for efficacy, the active searching behaviour of some EPNs would allow them to seek out and find *P. ficus* individuals in their cryptic habitats post-application onto foliage. It is also reasonable to assume that a foliage-adapted insect life stage would have no evolved defences against insect pathogens in the soil.

EPNs are soil-adapted organisms, with the prevalent environmental conditions outside of the soil posing challenges to their survival and efficacy. EPNs are sensitive to extremes of temperature (Grewal *et al.*, 1994), relative humidity (%RH) (Glazer, 1992), and ultraviolet radiation (UV) (Gaugler *et al.*, 1992), with individual environmental requirements varying between species. However, the limitations involved can be manipulated – for example, nematode species can be applied in greenhouses, where the temperature and humidity can be controlled, or applied to crops in the field at points in the day when the temperature and humidity most closely match the optimal conditions for the nematode species used. Additionally, adjuvants can be used as co-formulants to change the properties of EPN treatments. Adjuvants are defined as additives that can either augment or modify the effects of crop treatments (Krogh *et al.*, 2003).

Research into the use of EPNs in controlling South African mealybug species is ongoing. In Van Niekerk and Malan's (2012) assessment of the efficacy of EPNs against the citrus mealybug, *Planococcus citri* (Risso), it was found that both *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005 and *Heterorhabditis zealandica* Poinar, 1990 were able to infect and complete their life cycles in adult female *P. citri*. The first 2 to 4 h post-application were also established as being critical for the optimal infection of *P. citri*, and that humidity was the main limiting factor preventing IJs from surviving long enough to infect their host. Further research by Van Niekerk and Malan (2013) investigated the ability of adjuvants to improve EPN formulations for the infection of *P. citri*, finding that the addition of spreader and sticker adjuvants both increased the pathogenicity of *H. zealandica* and the deposition of nematodes on leaves.

Stokwe and Malan (2016) tested EPNs for use against another South African mealybug pest, the obscure mealybug *Pseudococcus viburni* Signoret, 1875, which is a major pest of pome fruit. They found that EPNs (specifically *H. zealandica* and *S. yirgalemense*) were able to penetrate and complete their life cycle within adult *P. viburni* females. The ability of EPNs to penetrate cryptic habitats so as to infect their insect hosts was also demonstrated, with IJs of *H. zealandica* being able to enter infested apple cores in order to infect *P. viburni* individuals within.

Finally, Le Vieux and Malan (2013) assessed the potential of EPNs in controlling the vine mealybug. Eight EPN species, six of which are indigenous to South Africa, were screened for pathogenicity to *P. ficus*, with the two species proving to be the most effective being the indigenous species, *H. zealandica* and *S. yirgalemense*. Later research by Le Vieux and Malan (2015) indicated that *S. yirgalemense* was capable of detecting *P. ficus* individuals, and of moving towards them for purposes of infection, with both traits being of use in foliar application.

The main objective of the current study was to determine the potential of three new locally isolated EPNs species to control *P. ficus* females under laboratory conditions. The mortality and penetration rate of the infective juveniles (IJs) was determined. *Steinernema yirgalemense* was used for further laboratory analysis with regard to penetration time, temperature and humidity, aimed at above-ground application on grapevine.

MATERIALS AND METHODS

Source of nematodes

Nematode species used in this study (Table 2.1) originated from soil samples collected locally, which were maintained and cultured at Stellenbosch University. IJs were cultured *in vivo* by means of using larvae of the mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Inoculated mealworms were kept at 25°C until IJ emergence, before being transferred to modified White traps (Woodring & Kaya, 1988). The IJs harvested from White traps were transferred to vented culture flasks and stored at 14°C, in keeping with the guidelines set out by Kaya and Stock (1997). The flasks were gently agitated weekly so as to improve their aeration, and the IJs were used within one week of emergence.

Table 2.1. Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) used by species, isolate, habitat, locality, and GenBank accession number, noting the length and maximum body width of the infective juveniles involved.

Species	Strain	Habitat	Locality	Accession Number	Length of IJ (µm)	Width of IJ (µm)
H. noenieputensis	SF669	Fig tree	Noenieput, Northern Cape	JN620538	528 (484–563)	21 (19–23)
S. jeffreyense	J194	Guava tree	Jeffrey's Bay, Eastern Cape	KC897093	924 (784–1043)	35 (23–43)
S. yirgalemense	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295	635 (548–693)	29 (24–33)
Steinernema sp.	WS9	Litchi orchard	Nelspruit, Mpumalanga	KP325086	1054 (953–1146)	35 (29–41)

Source of insects

A laboratory culture of *P. ficus* was established to ensure reliable access to females. The culture originated at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij in Stellenbosch, South Africa, with it being propagated on butternut squash in a Perspex cage, under ambient conditions. The cage was vented with mesh netting to allow for air circulation, but otherwise kept sealed, so as to prevent the escape of small crawlers. A fresh butternut was added once every three weeks to allow the individuals involved to migrate from the older butternut, which was then removed once rot had begun to set in. Female mealybugs were handled with a paintbrush and tweezers. Individuals were removed only if they were not currently feeding, as damage to mouthparts can negatively impact the insect's survivability.

Bioassay protocol

A 24-well bioassay tray was used as the test arena for inoculating mealybugs with EPN species. Twelve alternate wells per tray were used. A 13-mm diameter piece of filter paper was added to each alternate well, to absorb any excess liquid. One female *P. ficus* was added to each filter paper-lined well, for a total of 12 individuals per tray. The individuals were then treated with 50 µl of nematode-containing suspension, which was applied directly to the filter paper of each well, using a pipette. The trays were then covered with a glass cover to prevent the insects escaping. The bioassay tray was

covered with the lid, and the whole tray was secured with an elastic band. One of the trays was considered to be a single replicate. Five replicates (with 60 insects per treatment) each were kept in plastic containers, lined with moist paper towels, to maintain humidity levels of > 95%. The containers were closed with their lids and kept in growth chambers at 25°C for 48 h, after which they were removed, with individual mealybugs being assessed for mortality and infection. The mealybug cadavers were then removed from their wells, and placed into Petri dishes, lined with moistened pieces of filter paper. The Petri dishes were then sealed with Parafilm, and incubated at 25°C for another 48 h to allow for nematode development to take place inside the insects.

Pathogenicity and penetration

The bioassay protocol described was used to determine the pathogenicity of four EPN species towards *P. ficus* (Table 2.1). Mealybugs in each treatment were inoculated with 100 IJs in 50 µl of water. Mealybug mortality was assessed after 48 h, with such mortality being defined as a lack of movement under the exertion of light external pressure, and a change from the mealybug's normal colour. The cadavers were dissected using a microscope (Leica MZ7s) to establish the presence of nematodes. A droplet of water was placed onto the cadaver on a clean Petri dish, in which the cadaver was dissected, with the nematodes contained within being counted. The experiment was repeated on a later date with a fresh batch of nematodes.

Infection rate

Five Petri dishes (of 9 cm diam.) were lined with pieces of filter paper, whereupon six female mealybugs were placed in each. Nematodes were applied in 1 ml of water, containing 800 IJs of *S. yirgalemense*. Each dish was sealed with Parafilm, and then placed in an incubator at 25°C. Each of the five treatments was removed from the Petri dish after 30, 60, 180, 240, or 480 min, rinsed with water, and then placed in a clean Petri dish lined with a piece of moistened filter paper. The Petri dishes were then sealed with Parafilm and returned to the incubator for 48 h, after which mortality was confirmed by means of dissection.

Effect of temperature

The effects of different temperatures on the mortality of female *P. ficus*, when treated with *S. yirgalemense*, were tested. Five 24-well bioassay plates were prepared per temperature treatment, as described in the bioassay protocol. *Steinernema yirgalemense* IJs were applied to each individual at a concentration of 100 IJs / 50 µl. One container was placed (five 24-well bioassay plates; 60 insects) inside an incubator at 15°C, 25°C, and 30°C. After 48 h, the mealybugs were assessed for mortality. The experiment was repeated on a different test date with a fresh batch of nematodes.

Effect of humidity

The effects of different levels of humidity on the mortality of female *P. ficus* individuals, when treated with *S. yirgalemense*, were tested. Three containers were prepared with solutions of glycerol (60% RH), KNO3 (80% RH), and moistened tissue paper (100% RH). Five 24-well bioassay plates were prepared per humidity treatment. Alternating wells of each plate were lined with pieces of filter paper, onto which one female *P. ficus* was placed, with 12 adults being positioned per plate. *Steinernema yirgalemense* were applied at a concentration of 100 IJs / 50 µl. Netting was glued to the surfaces of each plate, and a lid was placed inside each respective container. The plates were then secured with their lids, and placed inside an incubator at 25°C. After 48 h, the trays were removed, and the mealybugs inside were assessed for mortality. The experiment was repeated on a different test date.

Data analysis

STATISTICA statistical analysis, software version 13 (TIBCO Inc., 2017), was used to establish the variance estimation, precision and comparison (VEPAC), and for the analysis of variance (ANOVA). Bonferroni's method was employed for the post hoc comparison of means, with significant differences being calculated to the 95% probability level.

RESULTS

Pathogenicity and penetration

A factorial ANOVA analysis of the results showed no significant difference between the main effects of treatment and date, enabling the data from the two test dates to be pooled. A VEPAC analysis of the mortality caused by the four EPN species to the *P. ficus* investigated showed a significant difference between species ($F_{(4, 50)} = 5.8179$, $\rho = < 0.01$) (Fig. 2.1). *Heterorhabditis noenieputensis* caused 90% ± 3% mortality of female *P. ficus*, which was significantly higher than for the other three species. The next most effective was *S. yirgalemense* (63% ± 7%), followed by *S. jeffreyense* (40% ± 7%). Mortality caused by the *Steinernema* isolate WS9 (9% ± 4%) proved not to be significantly different from the control (2% ± 1%).

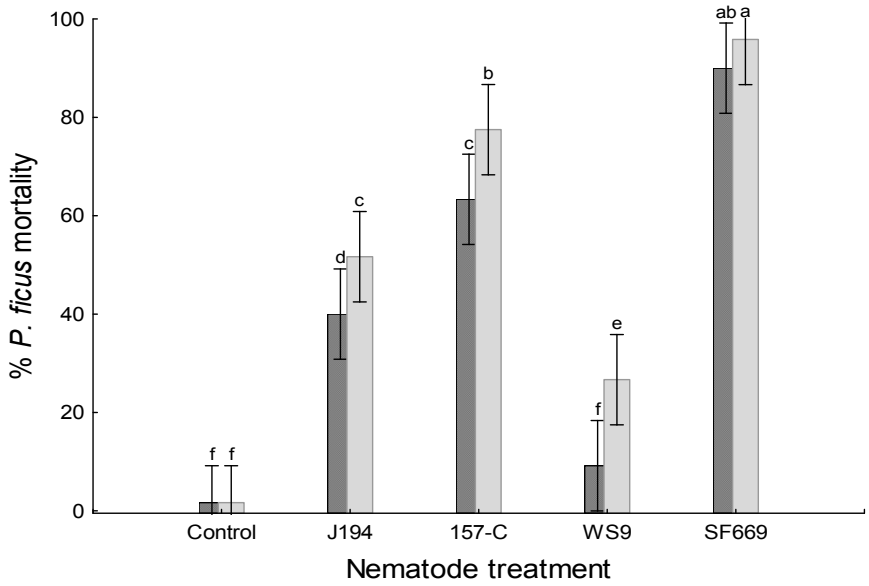

Figure 2.1. Mean percentage (95% confidence interval) mortality for female *Planococcus ficus*, 48 h ( ) and 96 h ( ) post treatment, using *Steinernema jeffreyense* (J194), *S. yirgalemense* (157-C), *Steinernema* spp. (WS9), and *Heterorhabditis noenieputensis* (SF669). Infective juveniles (IJ) were applied to *P. ficus* at a concentration of 100 IJs/insect and kept at 25°C (one-way ANOVA: $F_{(4, 50)} = 5.818$; $\rho < 0.005$). The means of bars sharing the same letter are not significantly different from each another.

Analysis using a one-way ANOVA of the penetration rate of IJs into *P. ficus* showed a significant difference between the number of nematodes found in mealybug cadavers ($F_{(3, 150)} = 3.4822$, $\rho = < 0.01$). However, on closer inspection, no significant difference was found between the mean number of nematodes found in *S. jeffreyense* (3.5 ± 0.4), *S. yirgalemense* (3.7 ± 0.5), and *H. noenieputensis* (4.3 ± 0.5). Apart from for *Steinernema* spp (WS9) (0.1 ± 0.3), all of the species tested were found to be in significantly higher numbers in the *P. ficus* cadavers (Fig. 2.2).

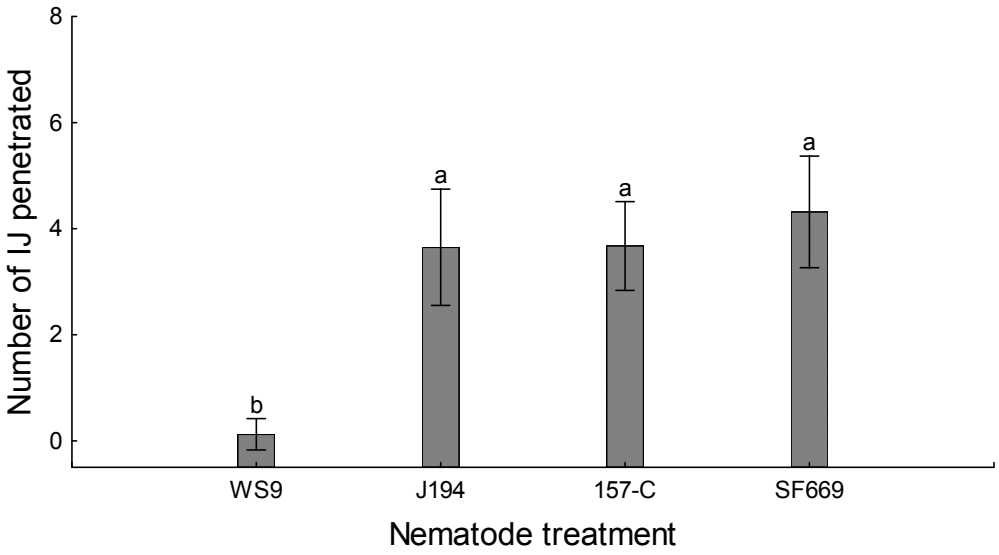

Figure 2.2. The mean number of nematodes (95% confidence interval) found within the cadaver of female *Planococcus ficus* post treatment with *Steinernema* sp. (WS9), *S. jeffreyense* (J194), *S. yirgalemense*, and *Heterorhabditis noenieputensis* (SF669) (one-way ANOVA: $F_{(3, 150)} = 3.482$; $\rho = 0.017$). Mealybugs were assessed for nematode penetration after 48 h exposure to infective juveniles (IJs). The means of bars sharing the same letter are not significantly different from each other.

Infection rate

The percentage mortality of *P. ficus* individuals exposed to *S. yirgalemense* at different time intervals was analysed using a one-way ANOVA. After a period of 30 min, 10% ± 7% mortality was encountered. with mortality between 40% and 60% being encountered after 8 h had elapsed. The percentage mortality showed significant increases from the 0.5-, 1- and 3-h intervals; however, no significant difference was observed in mortality after 3, 4 and 8 h ($F_{(4, 40)} = 3.4265$; $\rho = 0.02$) (Fig. 2.3).

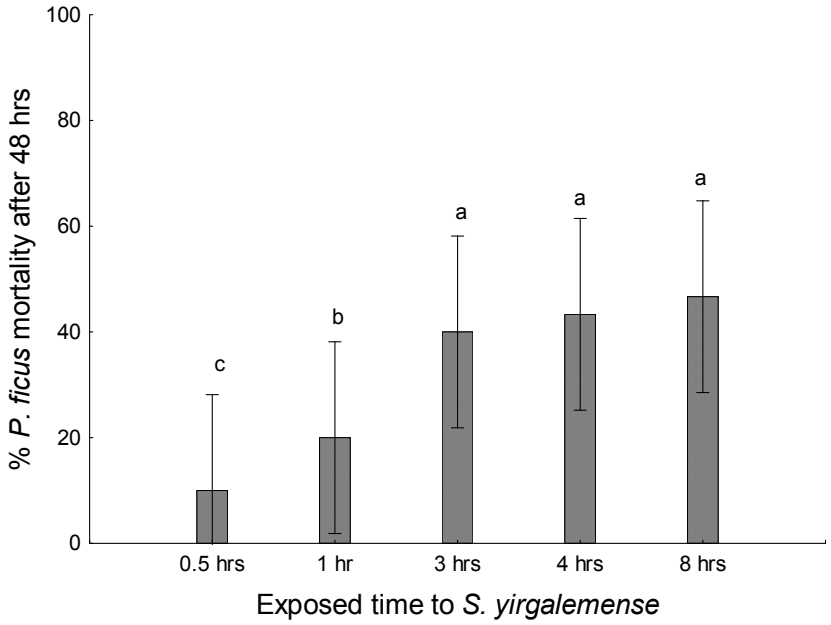

Figure 2.3. Mean percentage (95% confidence interval) mortality for female *Planococcus ficus* after exposure to *Steinernema yirgalemense* for different time intervals, at a concentration of 80 infective juveniles (IJs) / insect and mortality determined after 48 h. The means of bars sharing the same letter are not significantly different.

Effect of temperature

Factorial ANOVA analysis was used to compare the results of *S. yirgalemense* infection of *P. ficus* at different temperatures. The difference in mealybug mortality between the different temperatures was shown to be significant ($F_{(2, 48)} = 96.274$; $\rho < 0.01$). Mealybugs kept at 25°C resulted in the highest mortality (72% ± 3%), with the next most effective temperature being 30°C (45% ± 3%). Mealybugs treated with nematodes and kept at 15°C for 48 h resulted in the lowest mortality (9% ± 3%) (Fig. 2.4).

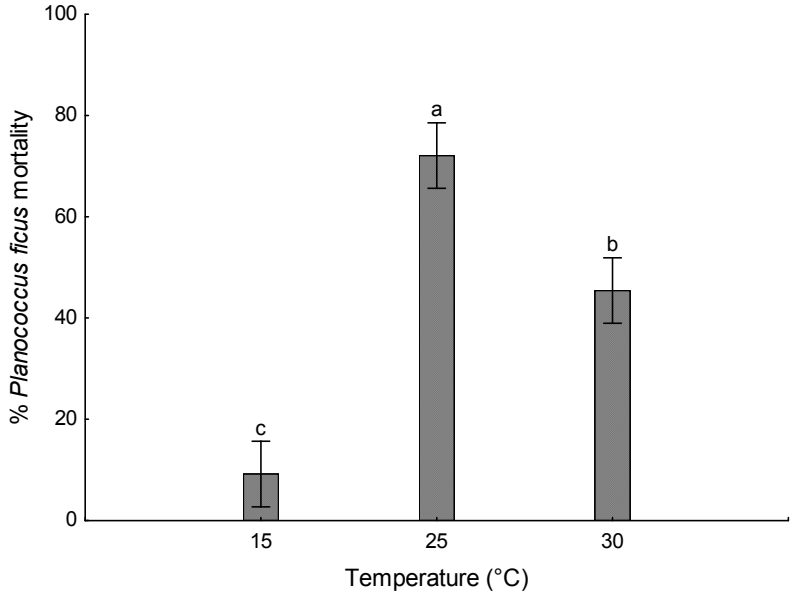

Figure 2.4. Mean percentage (95% confidence interval) mortality for female *Planococcus ficus* after exposure to *Steinernema yirgalemense* at different temperatures. IJs were applied at a concentration of 100 IJs / 50 µl and *P. ficus* was assessed for mortality after 48 h. The means of bars sharing the same letter are not significantly different.

Effect of humidity

A factorial ANOVA of female *P. ficus* inoculated with *S. yirgalemense* indicated a significant difference in the ability of *S. yirgalemense* to cause mortality in *P. ficus*, when kept in environments of differing relative humidity (RH) ($F_{(2, 53)} = 32.433$; $\rho = < 0.01$). *Steinernema yirgalemense* was most effective in causing mortality in *P. ficus* when kept at 100% RH (70% ± 3%), followed by at 85% RH (61% ± 3%), and then at 75% RH (40% ± 3%) (Fig. 2.5).

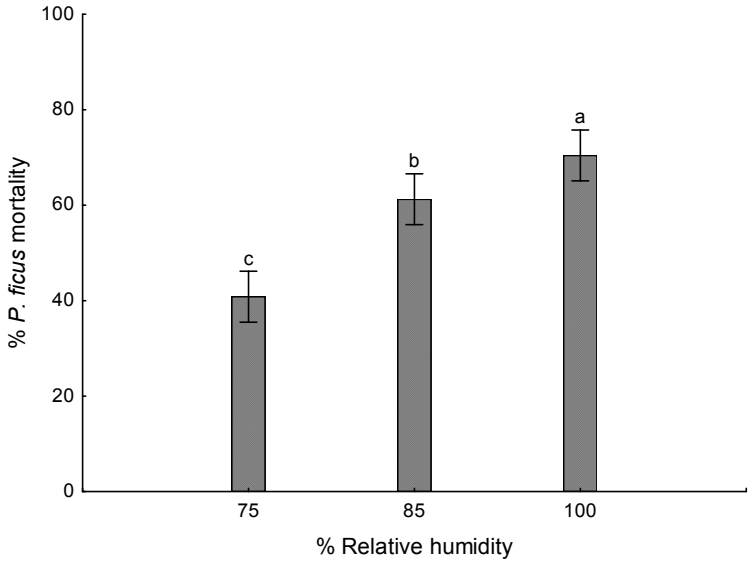

Figure 2.5. Mean percentage (95% confidence interval) mortality for female *Planococcus ficus* after exposure to *Steinernema yirgalemense* at different relative humidity. IJs were applied at a concentration of 100 IJs / 50 µl, and *P. ficus* was assessed for mortality after 48 h. The means of bars sharing the same letter are not significantly different.

DISCUSSION

Soil-based application of EPNs for the control of insect pests is logical, and effective EPN products are commercially available for the control of soil-based pest insect life stages. Foliar application of EPNs initially appears obvious, as EPNs have high pathogenicity to insects, high specificity, and, as living creatures, they are at an advantage when it comes to encountering the target pest, due to their ability to seek out prey. Different EPN species also have different levels of pathogenicity, as each has its own specific characteristics. Consequently, though EPNs can, by definition, not have adapted to prey specifically on pests of foliage, the IJs of EPN species are likely to possess traits that make them more or less effective against target pests. For this reason, it is important to identify which EPN species are the most suitable for use against a specific insect pest, and what the traits involved are.

The current study screened three described species, *viz*. *S. jeffreyense, Heterorhabditis noenieputensis,* and *Steinernema* spp., to compare their pathogenicity to adult female vine mealybugs to that of a previously screened species, *S. yirgalemense*. *H. noenieputensis* was shown to be the most effective against adult female *P. ficus*, causing mortality of 90% under ideal laboratory conditions. *Steinernema yirgalemense* was the next most effective, causing mortality of 63%.

The above is in keeping with an earlier study by Le Vieux and Malan (2013), who screened various EPN species for pathogenicity to adult female *P. ficus*. Eight EPN species overall, of which six were indigenous to South Africa, and two, *S. feltiae* and *H. bacteriophora*, which are commercially available, were screened for their ability to cause mortality in adult female *P. ficus*. Of the eight, the two most effective species were found to be *H. zealandica* and *S. yirgalemense*. The attainment of such a result was reflected in a study by Van Niekerk and Malan (2012), who screened six indigenous EPN species for pathogenicity to *P. citri*, finding *H. zealandica* and *S. yirgalemense* also to be most effective against *P. citri*, indicating that the EPN species concerned are highly effective against mealybugs.

Van Niekerk and Malan (2012) and Le Vieux and Malan (2013) selected *S. yirgalemense* instead of *H. zealandica*, due to the former's status as a commercially produced EPN species in South Africa, as well as to the difficulties posed to the mass culture of heterorhabditid species in liquid culture. Male *Heterorhabditis* are unable to copulate in liquid solution, which results in only one generation being produced in the case of hermaphrodites (Strauch *et al*., 1994; Ehlers & Shapiro-Ilan, 2005; Ferreira & Malan, 2014).

Each EPN species was also screened for their ability to penetrate adult female mealybugs. No significant difference was observed in the number of IJs of *S. yirgalemense* (3.7 ± 0.4 IJs per cadaver), *H. noenieputensis* (4.32 ± 0.5), and *S. jeffreyense* (3.5 ± 0.5) present in infected cadavers post-dissection, though the presence of each was significantly higher than was that of *Steinernema* spp. (WS9) (0.13 ± 0.1). This could be explained by the difference in size of the IJs of each species (Nguyen & Smart, 1995), with the IJs of *Steinernema* spp. WS9 being both longer (1054 µm, on average) and wider (35 µm) than either *S. yirgalemense* or *H. noenieputensis*, and longer than, but as wide as, *S. jeffreyense*.

Previous studies have compared the pathogenicity of one EPN species to different life stages (and, therefore, sizes) of the obscure mealybug *P. viburni*, finding that the smaller-bodied life stages were less susceptible to IJ infection than were the adults (Stokwe, 2009). Bastidas *et al.* (2014)

assessed the ability of four *Steinernema* species to persist in four small insect hosts, with each life stage concerned being less than 5 mm long. It was found that in general, the larger the IJ, the less successful it was in terms of infecting and completing its life cycle within the host. The relationship concerned is illustrated in the results of the screening in the current study, with the most successful species also being the smallest.

One advantage that the use of EPNs holds over chemical control methods is the former's ability to seek out their target pests in their cryptic habitats. However, the application of EPNs to insect life stages above ground has, typically, yielded variable results, depending on the species, the host species, and the environmental conditions involved (Begley, 1990; Gaugler *et al.*, 1992; Grewal *et al.*, 1994). Consequently, EPNs cannot be used indiscriminately against insect pests, and their environmental preferences must be established for their use to be most effective. In the current study, the speed at which *S. yirgalemense* achieved maximum mortality when applied to adult female *P. ficus*, as well as its temperature and humidity requirements for optimal performance, were assessed.

The infection rate assay was performed in order to determine the minimum amount of time required for IJs to locate and penetrate *P. ficus*, under optimal conditions (25°C, 100% RH). The present study showed that the IJs of *S. yirgalemense* were able to cause mortality in *P. ficus* after only 30 min, and that exposure to *S. yirgalemense* for longer than 3 h did not significantly improve mortality. Such a rate is comparable to the optimal time-to-mortality of *S. yirgalemense* against *P. citri* at 120 min (Van Niekerk & Malan, 2013). The rate attained would indicate that methods of application of *S. yirgalemense* on grapevine foliage, in controlling *P. ficus*, should aim to preserve optimum conditions for activity, mobility and infectivity for at least 3 h.

EPNs are highly sensitive to temperature, with each species possessing different optimal temperature niches for optimal IJ activity (Grewal *et al.*, 1994), due, in part, to the negative effect that high temperatures have on oxygen in solution (Wright *et al.*, 2005). The temperature study in this chapter was performed to determine the temperature at which *S. yirgalemense* is most able to infect and cause mortality in adult female *P. ficus*, under otherwise ideal conditions. *Steinernema*

yirgalemense IJs were found to cause highest mortality in *P. ficus* at 25°C (72%), followed by at 30°C (45%), with the mealybugs incubated at 15°C showing the lowest overall mortality (9%). The finding is in contrast to those made by Van Niekerk and Malan (2013), in which no significant difference was observed in *H. zealandica* efficacy, when applied to *P. citri* and incubated at 15°C, 20°C and 25°C. Compared to *H. zealandica*, *S. yirgalemense* can be inferred as being less tolerant of lower temperatures, with ideal temperature ranges for their use being around 25°C, and no higher than 30°C.

EPNs are soil-adapted, and so consequently require both high levels of relative humidity in order to infect their hosts (Lacey & Unruh, 1998), and a thin film of water to enable their movement (Wright *et al.*, 2005). In the current study, mealybugs were inoculated with *S. yirgalemense* and incubated at varying percentages of relative humidity. *Steinernema yirgalemense* were found to be most effective against *P. ficus* at 100% RH, giving 70% mealybug mortality. Such mortality was significantly higher than that which was attained with the lower percentage RH used. The finding concurs with those that were made in previous studies of the EPN's use against *P. citri* (Van Niekerk & Malan, 2013), which tested *S. yirgalemense* and *H. zealandica* against the citrus mealybug at differing water activity (a_w) levels, which were roughly equivalent. Both the current study and the study by Van Niekerk and Malan (2013) clearly illustrate that *S. yirgalemense* performs best at maximum moisture levels.

Conclusions can be drawn from the present study that both *H. noenieputensis* and *S. yirgalemense* are promising indigenous EPN candidates for the control of *P. ficus* on foliage, with the latter proving to be a more attractive candidate for future work, due to the project that is currently being undertaken into its *in vitro* mass liquid culture in South Africa. Future research should focus on attempts to mitigate the environmental weaknesses of *S. yirgalemense* on foliage, and its intolerance to extremes of temperature and low humidity, in order that it might persist on foliage long enough to find, infect, and cause mortality in, adult female *P. ficus*.

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CHAPTER 3

Adjuvants to improve the efficacy of *Steinernema yirgalemense* application against *Planococcus ficus* in a greenhouse environment

ABSTRACT

The vine mealybug (*Planococcus ficus*) is regarded as the dominant mealybug pest of grapevines in South Africa, with entomopathogenic nematodes (EPNs) being touted as a potential alternative to chemical pesticides in terms of their control, though their vulnerability to above-ground environmental conditions has limited their use. In this study, tests were conducted to assess the ability of the adjuvants to increase the amount of deposition of *S. yirgalemense* on grapevine leaves. The combination of Nu-Film-P® and Zeba® resulted in significantly more nematodes (30) being deposited per 4 cm^2 leaf disc than with either the control (14.8), or with Nu-Film-P® (23.3), though not significantly more than with Zeba® alone (29.2). The ability of *S. yirgalemense,* in conjunction with the two adjuvants, to control *P. ficus* on grapevine foliage was then assessed under controlled conditions. The application of *S. yirgalemense* with both Zeba® and Nu-Film-P® to *P. ficus* on leaf discs in the growth chamber resulted in 84% mortality, significantly greater than that attained by *S. yirgalemense* application with either Zeba® (47%), or water alone (26%). Similar results were observed in the glasshouse trial, in which the combination of *S. yirgalemense*, Zeba® and Nu-Film-P® offered 88% control of *P. ficus* on leaf discs hung in vineyards, compared with the control that was achieved with *S. yirgalemense* with either Zeba® (56%), or water alone (30%). This study demonstrates the potential of a combination of *S. yirgalemense* with adjuvants to control significant percentages of *P. ficus* on grapevine foliage, compared with using EPNs alone.

Key words: Entomopathogenic nematodes, EPNs, *Steinernema yirgalemense*, vine mealybug, *Planococcus ficus*, above-ground application, glasshouse, growth chamber, Zeba®, Nu-Film-P®

INTRODUCTION

South Africa is the twelfth largest producer of wine and table grapes in the world, producing 2.61% of the world's grapes in 2014 (FAO, 2016). Wine and table grape production is, therefore, of significant economic importance to South Africa, and especially to the Western Cape Province, where the majority of wine and table grape production occurs (SAWIS, 2015; SATI, 2016).

The vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) is a pest of grapevine that is found in most grape-producing regions worldwide (Ben-Dov, 1994; Walton & Pringle, 2004). It is the predominant pest of grapevine in South Africa (Walton, 2003; Walton *et al.,* 2004), causing damage via phloem feeding, the reducing of the flow of plant sap to the fruit, thereby reducing yield (Millar, 2002). Mealybugs also deposit waxy residues and sooty mould-encouraging honeydew, thereby disfiguring the grapes (Geiger & Daane, 2001), and transmitting the grapevine leafroll-associated virus type III (GRLaV-3), which causes rolling and discolouration of the leaves (Bovey *et al*., 1980).

Existing measures to control the vine mealybug on grapevines have tended to focus on chemical control, with chlorpyrifos (Walton, 2003), and in some cases dichlorvos or methidathion (Nel *et al*., 1999), being of particular note in South Africa. However, due to the potential for harm to non-target organisms via direct contact with, or the contamination of, groundwater, as well as the potential for target insects to develop resistance, biological alternatives are often sought as a possible solution to the existing problem (Hussaini, 2002). In particular, *P. ficus* has innate defences against chemical pesticides, such as its high reproductive rate allowing for an increase in the pace of development of its resistance to pesticides (Daane *et al*., 2008), while both its cryptic choice of environment (typically beneath raised grapevine bark) and the waxy filaments that it produces serve as barriers to pesticide contact, post-application (Berlinger, 1977).

Entomopathogenic nematodes (EPNs) are soil-based pathogens of insects, typically of the families Steinernematidae and Heterorhabditidae that are widely used as biocides against the soil-

based insect life stages (Campos-Herrera, 2015). EPNs encounter their prey by means of exhibiting behaviour on a continuum between stationary, 'ambushing' behaviour (which is better for active prey) and mobile, 'cruising' behaviour (which is more suited to passive and/or cryptic prey), while in a free-living infective juvenile (IJ) life stage (Lewis, 2002; Campbell *et al.*, 2003; Griffin *et al*., 2005). Once they encounter prey, the IJs enter the pest insect's body cavity through the natural openings, killing the insect, in conjunction with its symbiotic bacteria species, and undergoing several generations within the cavity of the insect (Griffin *et al*., 2005). EPNs are an attractive potential biological control agent, due to their initial virulence to the target pest, to their ability to actively seek out insect pests, and their relatively low persistence within the environment (Smits, 1996; Wilson & Gaugler, 2004). The use of EPNs to control insect pests is common and effective against soil-based insect pests (Wilson & Gaugler, 2004).

Application of EPNs to control the above-ground insect life stages is less common, mostly due to the abiotic factors concerned, chiefly temperature (Grewal *et al*., 1994), humidity (Lello *et al*., 1996; De Waal *et al*., 2013), and ultraviolet radiation (Gaugler & Boush, 1978). EPNs make use of water film on the leaves in humid environments to infect their insect prey, making the former more useful in tropical and/or rainy environments than in dryer ones, or at the time of day in which relative humidity is highest (Mráček, 2002).

Arthurs *et al*. (2004) performed a review of 136 published trials, each investigating the potential of EPNs against above-ground pests. They found that nematode efficacy depended on the target habitat, in order of highest efficacy from boreholes, through cryptic habitats, to exposed habitats. Relevant studies have also yielded results in the order of highest efficacy ranging from laboratory environments, through greenhouse environments, to field studies. The above-mentioned studies illustrate a general trend in more sheltered environmental conditions being best for the successful application of EPNs, with the more similar the above-ground environment is to the soil environment (i.e. in terms of providing shelter from such abiotic factors as sunlight and air flow) betokening the more successful the EPN application is likely to be.

One possible means of increasing EPN efficacy on foliage involves the improvement of EPN formulations by means of adjuvants, which are chemical additives that alter the physical properties of formulations. The addition of adjuvants against insect life stages on foliage has been promising in the case of boring pests, as the tunnel/borehole environments that such insect life stages offer provide shelter not only to the insect pests, but also, potentially, to EPNs. Shapiro-Ilan & Cottrell (2006) assessed the efficacy of EPNs against the lesser peach tree borer *Synanthedon pictipes* (Grote and Robinson), finding *S. carpocapsae* to be the most effective EPN in this respect. Further trials tested the effects of several adjuvant compounds on the survival and efficacy of *S. carpocapsae*. Shapiro-Ilan *et al*. (2010) assessed the effect of five potential adjuvants, including Anti-Stress®, Moisturin®, Nu-Film®, Shatter-Proof®, and Transfilm®, on the survival of *S. carpocapsae*, selecting Shatter-Proof® for future testing, due to it causing lower EPN mortality than did the other candidates. The researchers concerned then applied *S. carpocapsae* treatments to peach trees in the control of *S. pictipes*, formulated with and without Shatter-Proof®, both alone, and including a post-application treatment of latex paint, a moistened diaper, or a fire gel spray (Barricade®) applied to the treated area. Shapiro-Ilan *et al*. (2010) found that nematode-only treatments failed to control *S. pictipes*, and that applications of nematodes with Shatter-Proof® did not improve peach tree borer mortality, relative to the application of nematodes alone. However, they found that applying nematodes alone, with post-treatment application of Barricade®, was the only treatment to result in significantly higher control of *S. pictipes* than was achieved in the control. Further trials (Shapiro-Ilan *et al.*, 2016) assessed the efficacy of *S. carpocapsae* when combined with Barricade®, both as a 4% spray applied to cover, and as a 2% formulation spray containing *S. carpocapsae*. The 2% formulation with *S. carpocapsae* was found to offer significantly higher control of *S. pictipes* than did applications of water or nematodes alone, with the efficacy found being comparable to that which was attained with the application of chlorpyrifos.

The anti-transpirant Folicote® has been used to increase the lifespan of *S. carpocapsae* on beans, improving IJ viability from 38 to 60%, at 60% RH over 6 h in an exposed foliage environment

(Glazer, 1992). Baur *et al*. (1997) investigated the application of several adjuvant-nematode preparations for efficacy against the diamondback moth *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae), and concluded that, while such preparations probably did not justify their commercial application against *P. xylostella*, the addition of adjuvants improved the persistence and efficacy of the EPNs concerned. Head *et al*. (2004) found that addition of either of the two surfactants, Agral® and Triton X-100®, to formulations of *S. feltiae* significantly increased the latter's efficacy against the foliage-dwelling life stages of the tobacco whitefly, *Bemicia tabaci* (Gennadius) (Homoptera: Aleryodidae), on tomato and verbena plants, with no adverse effects occurring, either to the EPNs, or in the form of any phytotoxic effects to the host plants.

The main objective of the current study was to test the effect of adjuvants on the improvement of the above-ground application of EPNs to control *P. ficus* on grapevine. Such testing was accomplished by investigating the improvement of nematode depositions on leaves with the addition of adjuvants. Above-ground applications of EPNs to control *P. ficus* were further investigated by means of applying the nematodes, together with an adjuvant, in a growth chamber, followed by an investigation under natural conditions, in the form of a greenhouse bioassay.

METHODS AND MATERIALS

Source of nematodes

The nematode species, *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, used in the current study originated from samples that were collected locally, maintained and cultured at Stellenbosch University. IJs were cultured *in vivo* by means of infecting larvae of the mealworm beetle *Tenebrio molitor* L. (Tenebrionidae: Coleoptera) with IJs. Infected mealworms were kept at 25°C until IJ emergence, before being transferred to White traps (White 1927). The IJs harvested from the White traps were transferred to vented flasks, where they were kept at 14°C, in keeping with the guidelines set out by Kaya & Stock (1997). The flasks concerned were gently agitated once a

week to improve aeration. IJs for the experimentation were used within one week of emergence. The experiment was repeated on a different test date, with a fresh batch of nematodes.

Source of insects

A laboratory culture of *P. ficus* was established to ensure reliable access to adult female individuals. The culture, which originated at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij in Stellenbosch, South Africa, was propagated on butternut squash in a Perspex cage under ambient conditions. The cage was vented with mesh netting to allow for air circulation, although it was otherwise kept sealed to prevent the escape of any mealybug nymphs. A fresh butternut was added once every three weeks to allow the individuals to migrate from the older butternut, which was then removed, once rot had begun to set in. The adult female individuals were handled with a paintbrush and a pair of tweezers. Individuals were removed only if they were not currently feeding, as damage to mouthparts can impact the survivability of the insect.

Adjuvant deposition

An experiment was set up to test the efficacy of two adjuvants, Zeba® and Nu-Film-P®, in applying *S. yirgalemense* to grapevine leaves. Four nematode suspensions were set up, each containing 1000 IJs/ml, with one containing Zeba® (0.3 g/L), one containing Nu-Film-P® (0.6 ml/L), one containing equal parts Zeba® and Nu-Film-P® at 0.3g/L and 0.6ml/L respectively, and with a control of IJs in water alone.

A handheld sprayer was used to apply the above-mentioned formulations to the grapevine leaves. In the experiment, green grapevine leaves were used within 24h of harvest. Each suspension was applied to a grapevine leaf that was suspended from a line, from a distance of 20 cm, until runoff. The procedure was repeated, using five leaves per treatment. Each leaf was allowed 3min post-application to allow for any excess formulation to run off, after which time two 4 cm^2 discs were cut from each leaf, giving 10 discs per suspension. Each disc was then rinsed with 5 ml of tap water, which was collected in the individual wells of a bioassay tray. The nematodes present in the tap water

post-rinsing were counted, and compared between treatments. The experiment was repeated on a different test date with a fresh batch of nematodes.

Growth chamber bioassay

To simulate greenhouse conditions, large plastic containers were filled with water and placed at the bottom of growth chambers to increase the prevailing humidity. Grapevine leaves obtained from Welgevallen Experimental Farm were washed in a solution of water and 0.01% household bleach, rinsed thoroughly in tap water, and left to dry before use. Eight mealybugs were transferred to each of eight leaves (8 replicates, 64 insects) for each treatment. The leaves were cut to fit 13-cm-diameter Petri dishes lined with most filter paper. Treatments were water only; *S. yirgalemense* in water; *S. yirgalemense* + Zeba®; and *S. yirgalemense* + Zeba® + Nu-Film-P®.

Nematodes were applied to the leaves with the aid of a calibrated handheld spray applicator, at a concentration of 3000 IJs/ml. Zeba® and Nu-Film-P® were used in the treatments, at a concentration of 0.03% and 0.06%, respectively. The treatment formulations were prepared 1h before each trial. The leaves were left for 3min after treatment to eliminate excess runoff. They were then placed in small pockets, made of fine-mesh netting. The pockets were hung in a randomised block design in the growth chamber. After 48h, the mealybugs were removed from the leaves, and mortality was assessed. The mealybugs were then washed to remove surface nematodes, and placed in Petri dishes, lined with moistened filter paper, and incubated for a further 48h at 25°C. The temperature and humidity were monitored using I-buttons, which were placed inside the growth chambers. The experiment was repeated on a later date with a fresh batch of nematodes.

Greenhouse trial

The leaf disc pockets, mealybugs, and nematode/adjuvant solutions were prepared as for the previously described growth chamber bioassay, with the same treatments and number of replicates per treatment being used. After preparation, each of the 40 pockets containing the treated mealybugs was hung on Chenin Blanc potted grapevines located in a glasshouse. The temperature and humidity

in the glasshouse were monitored using data loggers. The experiment was repeated at a later date, with the results being pooled for analysis.

Data analysis

The analysis of all trial data was conducted using the STATISTICA statistical analysis software version 13 (TIBCO Inc., 2017). The data were analysed using variance estimation, precision and comparison (VEPAC) and analysis of variance (ANOVA), using Bonferroni's method for the post-hoc comparison of means. Significant differences were calculated to 95% probability level.

RESULTS

Adjuvant deposition

The two-way ANOVA that was used to analyse the main effects experienced during the tests indicated no significant difference between date and treatment, so that the data from the two test dates were able to be pooled. The number of IJs that were present in the samples retrieved from the grapevine leaves were analysed using a one-way ANOVA, whereupon a significant difference was observed between treatments ($F_{(3,76)} = 11.548$, $p = <0.01$). The combination of Nu-Film-P® and Zeba® was seen to result in the deposition of a significantly higher number ($p = 0.01$) of IJs (30.8 ± 4 IJs/4 cm^2) than did Nu-Film-P® alone (23.3 ± 2 IJs/4 cm^2), in comparison to the control (14.8 ± 2 IJs/4 cm^2). However, the combination of Nu-Film-P® and Zeba® did not result in significantly more nematodes being deposited ($p = 0.59$) than did the Zeba® alone (29.2 ± 3 IJs/4 cm^2) (Fig. 3.1).

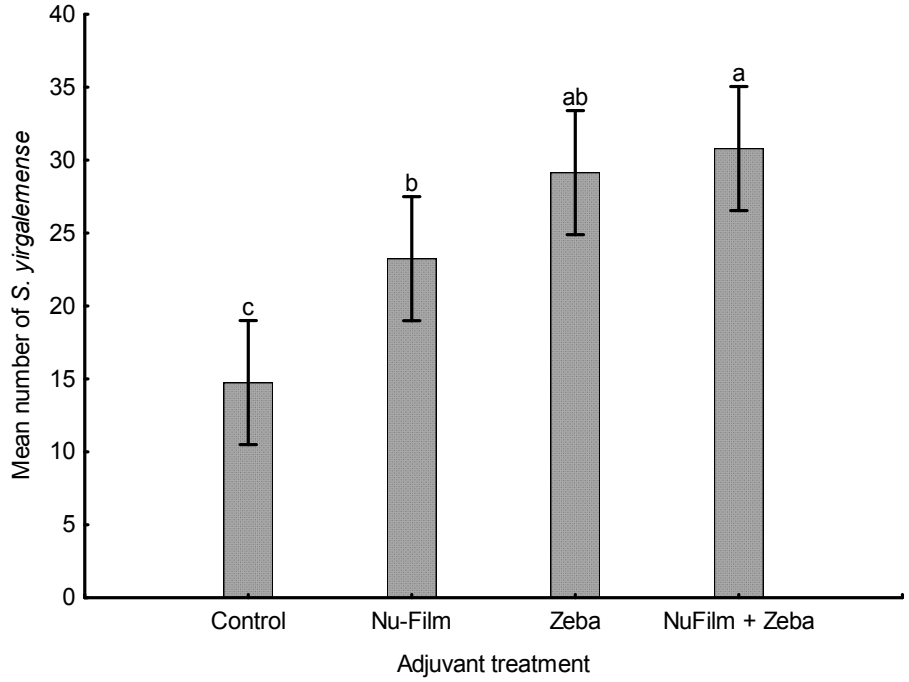

Figure 3.1. Mean percentage (95% confidence interval) deposition of *Steinernema yirgalemense* infective juveniles (IJs) onto grapevine leaves, applied with a handheld sprayer, at a concentration of 1000 IJs/ml. After rinsing the leaves with tap water, the nematodes in the runoff were counted (one-way ANOVA: $F_{(3, 76)} = 11.548$, $p = <0.01$). Means of bars sharing a letter are not significantly different from one another.

Growth chamber bioassay

A two-way ANOVA, used to analyse the main effects of the study, indicated no significant difference between date and treatment, leading to the pooling of the data from the two test dates. An ANOVA analysis of *P. ficus* mortality caused by growth chamber treatments after 48 h showed that each treatment differed significantly from all others ($F_{(3,120)} = 241.52$, $p = < 0.01$). The combination of Zeba® and Nu-Film-P® was found to be the most effective (84 ± 5% mortality). The aforementioned combination was followed by Zeba® alone (47 ± 3%), and then by the nematodes alone (26 ± 2%), compared with the water control (9% ± 2%) (Fig. 3.2).

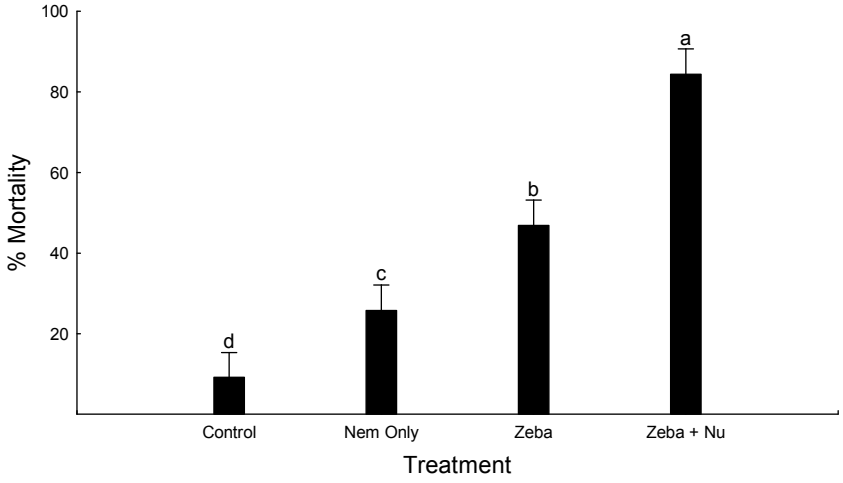

Figure 3.2. Mean percentage (95% confidence interval) mortality of *Planococcus ficus* on grapevine leaves, in a glasshouse environment, treated with *Steinernema yirgalemense* infective juveniles (IJs). IJs were applied to leaves with a handheld sprayer at a concentration of 3000 IJs/ml (one-way ANOVA: $F_{(3,120)} = 241.52$; $p = <0.01$). Means of bars sharing a letter are not significantly different from one another.

Greenhouse bioassay

A two-way ANOVA used to analyse the main effects indicated no significant difference between date and treatment, leading to the pooling of the data from the two test dates. An ANOVA analysis of mortality caused by growth chamber treatments after 48 h showed that each treatment differed significantly from all others ($F_{(3,120)} = 207.42$, $p = <0.01$). The combination of Zeba® and Nu-Film-P® was the most effective (88% ± 3% mortality), followed by Zeba® alone (56% ± 5%), and then by the nematodes alone (30% ± 3%), compared with the water control (13% ± 2%) (Fig 3.3).

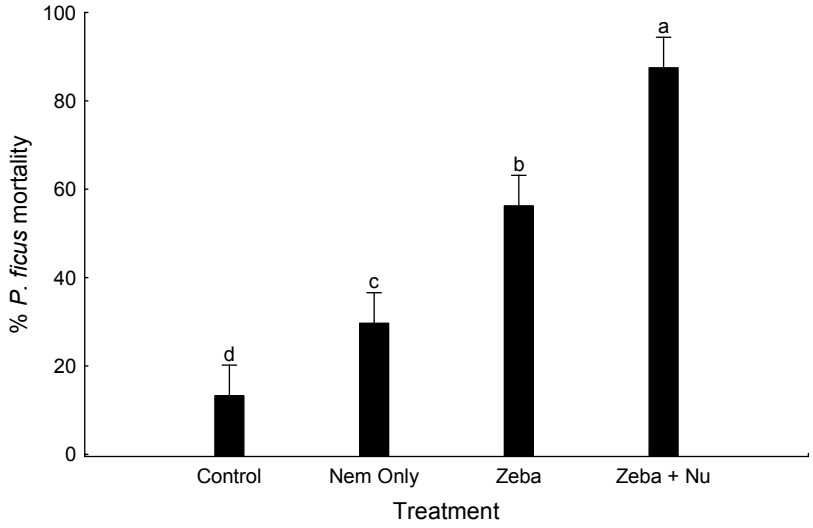

Figure 3.3. Mean percentage (95% confidence interval) mortality of *Planococcus ficus* on grapevine leaves kept in a greenhouse environment, post treatment with *Steinernema yirgalemense*. Infective juveniles (IJs) were applied to leaves with a handheld sprayer at a concentration of 3000 IJs/ml. Means of bars sharing a letter are not significantly different from one another.

DISCUSSION

Although *P. ficus* was found to be highly susceptible to EPNs in the laboratory bioassays, soil is their natural habitat, and special challenges are encountered under above-ground environmental conditions. Of such conditions, the most important is the moisture that has to be dealt with for the control concerned to be successful. One option for overcoming the problem of humidity is the addition of adjuvants to the nematode suspension, assisting in the ability of nematodes to stick onto the leaves involved, and prolonging the film of water on the leaves that is required for nematode movement.

The adjuvant that was found to be the most effective in improving the mortality of *P. ficus* was Zeba®, resulting in significantly higher deposition of *S. yirgalemense*, both alone and in combination with Nu-Film-P®. The treatment of Zeba® and Nu-Film-P® gave significantly better control than did Nu-Film-P® alone, although all the treatments resulted in significantly higher deposition of *S. yirgalemense* IJs onto grapevines than did water alone. The ability to double the number of IJs deposited onto grapevine leaves post-application makes Zeba® (and, to a lesser extent, Nu-Film-P®) an attractive addition to nematode application suspensions. The finding follows a similar trend in the research of Van Niekerk and Malan (2015), who assessed the use of Nu-Film-P® and Zeba® for the deposition of *H. zealandica* onto citrus leaf discs. In the study concerned, only the combination of Nu-Film-P® and Zeba® significantly increased the nematode deposition on citrus leaves, compared to the control, due to the waxy (water-repellent) coating on the citrus leaves. The finding indicates that Zeba® and Nu-Film-P® can more effectively be used when targeting plants without waxy coatings, such as grapevine leaves.

The results of the growth chamber bioassay showed that *S. yirgalemense* was most effective under optimal conditions (25°C, 100% RH), when applied to adult female *P. ficus* in a combination of Zeba® and Nu-Film-P®, with 84% mortality having been caused after 48 h. Such treatment was significantly more effective than were the *S. yirgalemense* and Zeba®, the *S. yirgalemense* with water, and the water-only control. The above indicates that, despite the fact that the addition of Nu-Film-P®

did not significantly improve the deposition of nematodes onto grapevine leaves over the application of Zeba® alone, it still does have a significant contribution to make to the successful infection of *P. ficus* by *S. yirgalemense*. Van Niekerk and Malan (2015) performed a similar bioassay, assessing the mortality of *P. citri,* post-application of *H. zealandica* and *S. yirgalemense* in suspension with distilled water, xanthan gum, or Zeba®. They found that the addition of Zeba® caused a significant increase in the mortality of *P. citri*, improving the *H. zealandica*-induced mortality by 22%, and the *S. yirgalemense* mortality by 27%, at 80% relative humidity.

The greenhouse bioassay sought to assess the impact of a less controlled environment on treatments from the growth chamber bioassay. However, unlike the growth chamber bioassay, the average temperature and humidity were much lower at 19°C and 59% RH over the course of the experiment. Interestingly, such conditions did not appear to lower the overall *P. ficus* mortality, following the trend set by the growth chamber bioassay, in which the most effective treatment was also that of Zeba® and Nu-Film-P®, followed by Zeba®, and by the IJs only, and being the least effective with water only. The mortality of the control mealybugs was higher in the greenhouse bioassay than it was in the growth chamber bioassay, although only by 4%, making this a promising indication that Zeba® and Nu-Film-P® can be used in conjunction to control *P. ficus* on grapevines under sheltered, or covered, conditions. The results was mirrored by Van Niekerk (2012), who emulated greenhouse conditions by performing a growth chamber bioassay to 22°C and 75 ± 8% RH. The researcher found that the addition of both Zeba® and Nu-Film-P® to *S. yirgalemense* was able to cause higher mortality in *P. citri* than did any other combination of *S. yirgalemense* or *H. zealandica* with water, Zeba® and Nu-Film-P®, aside from for *S. yirgalemense* alone.

In conclusion, the results obtained indicate the potential for *S. yirgalemense* to be used to control *P. ficus* on foliage under controlled conditions, which is a key step in developing methods to apply *S. yirgalemense* to *P. ficus* in the field. Zeba®, a polysaccharide starch, improves nematode deposition and infectivity when compared with Nu-Film-P®. The use of EPN suspensions containing Nu-Film-P® (a spreader and sticker) alone showed much lower improvement in *P. ficus* mortality

when compared with the use of suspensions containing Zeba® alone. However, combinations of both adjuvants offered significantly higher mortality, indicating that both adjuvants work synergistically to promote EPN survival and infectivity on foliage. When assessing adjuvants for use in EPN solutions going forward, attention must be paid to the qualities of each constituent, and how they interact. Additionally, the ability of suspensions of *S. yirgalemense*, Zeba® and Nu-Film-P® to achieve 88% mortality in *P. ficus* in the glasshouse warrants future research into the ability of *S. yirgalemense* to control other insect pests in indoor environments.

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CHAPTER 4

Foliar application of *Steinernema yirgalemense* to control *Planococcus ficus* in a South African Vineyard

ABSTRACT

The vine mealybug, *Planococcus ficus* Signoret (Hemiptera: Pseudococcidae), is a key insect pest of South African grapevine. The ability of mealybugs to avoid or resist the action of chemical pesticides has led to the investigation of alternative control methods, such as the application of entomopathogenic nematodes (EPNs). However, EPN application faces challenges, due to the maladaptation of EPN species to above-ground conditions. In this study, the ability of adjuvants to improve the control of *P. ficus* in grapevine using an indigenous nematode species, *Steinernema yirgalemense*, was investigated. In a semi-field trial, the combination of adjuvants Zeba® and Nu-Film-P® resulted in 66% control of *P. ficus* after 48h, compared with the use of Zeba® alone (43%), and EPNs alone (28%). Additionally, lower concentrations of EPNs showed predictably lower mortality rates of *P. ficus*. A trial was performed to assess EPN survival on grapevine foliage, when applied in the morning (high humidity / low temperature) compared with in the afternoon (high temperature / low humidity). Significantly, higher EPN survival was recorded at each time interval in the morning, compared with the same interval in the afternoon. This study demonstrates the ability of *S. yirgalemense*, when applied with adjuvants and at an appropriate time of day, to control *P. ficus* on grapevine, under semi-field conditions.

Key words: grapevine, vine mealybug, Steinernematidae, above-ground, Zeba®, Nu-Film-P®, *Steinernema yirgalemense,* entomopathogenic nematodes

INTRODUCTION

Mealybugs are scale insects of the family Pseudococcidae, notable for the waxy excretion that covers the bodies of the nymphs and females (Downie & Gullan, 2004). They are also important pests of South African viticulture (Annecke & Moran, 1982). Some such pests are the obscure mealybug *Pseudococcus viburni* on pome fruit (Wakgari & Giliomee, 2004), the citrus mealybug *Planococcus citri* on citrus (Hattingh *et al.*, 1998), and the vine mealybug on grapevine (Walton, 2003; De Villiers & Pringle, 2007).

The vine mealybug, *Planococcus ficus* (Signoret), causes damage to grapevines by feeding on phloem, diverting resources from fruit production, and reducing yield; by producing honeydew, which encourages the growth of sooty mould; and by serving as a vector for such diseases as vine leafroll associated closterovirus-3 (GLRaV-3) (Cabaleiro & Segura, 1997; Millar, 2002). *Planococcus ficus* is the pre-eminent mealybug pest of grapevines in South Africa, being able to feed on all parts of the vine at various times of the year, producing more honeydew, and having a faster generation time (with more eggs laid and faster development) than do similar species (Daane *et al.*, 2003, 2008). Populations of *P. ficus* undergo seasonal migration on grapevine, moving upwards to the branches and leaf buds as the temperature increases in spring and summer, and receding downwards onto the trunk in cooler months (Walton, 2003). The cryptic lifestyle of the vine mealybug (residing in crevices and under raised grapevine bark), as well as the hydrophobic waxy coating covering nymphs and females, prevents effective contact with insecticides, thus posing problems for control by means of traditional chemical methods (Walton & Pringle, 2004b).

Entomopathogenic nematodes (EPNs) are soil-based rhabditid roundworms, typically of the families Steinernematidae and Heterorhabditidae, which are characterised by their parasitism of soil-based insect life stages (Adams & Nguyen, 2002). EPNs exist in the soil as infective juveniles (IJs), which are the free-living survival stage. The IJs encounter prey insect life stages in the soil, utilising behaviour that falls on a spectrum between the extremes of active ‘cruising’ and passive ‘ambushing’ to locate their prey (Lewis *et al*., 1992, 1993). Once the IJ has come across its host, it either enters

the body of the insect through the latter's natural openings (the mouth, anus, or spiracles) or, in the case of some heterorhabditid species, using a dorsal tooth to penetrate soft parts of the cuticle directly (Dowds & Peters, 2002; Forst & Clarke, 2002). IJs carry within them symbiotic bacteria (*Xenorhabdus* spp. in the case of *Steinernema*, or *Photorhabdus* in the case of *Heterorhabditis*), the cells of which are retained in the IJ's intestine (Adams & Nguyen, 2002).

Once inside the haemocoel of the insect, the IJ releases the above-mentioned bacteria, which consume the tissues of the insect and multiply rapidly, killing the insect of septicaemia within a period of 1 to 2 days (Adams & Nguyen, 2002). The resulting mass of bacteria produces enzymes that convert the internal organs of the insect into a form that is suitable for consumption by the IJ, which consumes both the bioconverted insect and the proliferating bacteria (Forst & Clarke, 2002). Once food is plentiful, IJs develop into adult stages and reproduce. The first generation of heterorhabditids (and at least one steinernematid species) produce self-fertile hermaphrodites, with subsequent generations additionally producing males and females, which reproduce amphimictically (Griffin *et al*., 2005). By contrast, most Steinernematid species produce males and females from the first generation, and reproduce amphimictically for all their subsequent generations (Poinar, 1990). Food resources within the body cavity of the insect are limited, so that, once the available nutrients become inadequate, or conditions become overly crowded, the juveniles halt their growth and turn into IJs, keeping the cuticle of their previous stage as a protective sheath, before migrating from the body of the insect and starting the process anew (Griffin *et al*., 2005).

The ability of EPNs to cause mortality in insects has led to significant interest in their use as potential biocontrol agents, with several products having been developed and used successfully in the control of subterranean pest insect life stages (Wilson & Gaugler, 2004). However, attempts to apply EPNs for the control of foliage-based pest insect life stages have been considerably less successful than the above (Shapiro-Ilan *et al*., 2006; Chapter 1). EPNs are soil-inhabiting organisms that are intolerant to (various degrees of) excessive temperature (Grewal *et al*., 1994; Wright *et al*., 2005), to exposure to UV radiation (Gaugler & Boush, 1978; Gaugler *et al*., 1992), and to insufficient levels

of humidity (Glazer, 1992; Glazer *et al*., 1992a,b). IJs also rely on a thin film of water for mobility, and desiccation inhibits the nematodes' ability to find prey (Norton, 1978; Glazer, 2002). The characteristics mentioned severely limit the use of EPNs to control insect life stages when applied to foliage, as the reduced survival and mobility inhibit the former's ability to locate and infect the targeted pest. Additionally, the tolerance of each EPN species to each of these environmental factors varies, based on the species concerned (Glazer, 1992). As such, EPN application on the pests of foliage has yielded mixed results, with EPNs being most successfully used on pests in sheltered or cryptic habitats, including undercover conditions or in the glasshouse, and in the boreholes of the leaf-mining, or stem-boring, insect life stages (Arthurs *et al.*, 2004).

The improvement of pesticide application, be it chemical or biological, has tended to focus on such areas as application technology (Georgis, 1990; Lello *et al*., 1996; Beck *et al*., 2014) and the addition of adjuvants, consisting of chemicals that alter the physical properties of pesticide treatments. Adjuvants that have commonly been used, with success, to enhance EPN applications on foliage include thickeners, surfactants, evaporation retardants, and antidesiccants (Webster & Bronskill, 1968; MacVean *et al*., 1982; Shapiro *et al*., 1985; Glazer *et al*., 1992a; Head *et al*., 2004; Schroer & Ehlers, 2004). A metastudy by Arthurs *et al.* (2004) assessed existing studies on the efficacy of EPNs, in which it was established that the addition of adjuvants to EPN solutions improved deposition onto foliage (Mason *et al*., 1998), as well as survival and control, compared with the application of water alone (MacVean *et al*., 1982; Shapiro *et al*., 1985; Glazer *et al*., 1992a,b). The adjuvants mentioned have shown promise in increasing the efficacy of foliar EPN applications, although their commercialisation remains slow (Arthurs *et al.*, 2004; Shapiro-Ilan *et al*., 2006).

Previous research, including that of Van Niekerk & Malan (2012), has assessed the ability of EPNs to control South African mealybugs. The two researchers in question compared the efficacy of two indigenous EPN species, *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams and *Heterorhabditis zealandica* Poinar, in controlling populations of the citrus mealybug *Planococcus citri* Risso, an important pest of South African citrus. EPN treatments, both with and

without adjuvants, were formulated and applied to *P. citri* females in the greenhouse and under semi-field conditions. It was found that the addition of Zeba®, a superabsorbent polymer based on cornstarch, was able significantly to increase the ability of *S. yirgalemense* to cause mortality in female *P. citri* by protecting the EPNs from the prevailing environmental conditions in a semi-field trial.

Le Vieux & Malan (2013, 2015) examined the ability of *S. yirgalemense* and *H. zealandica* to control *P. ficus* in the soil, the given EPN's established ability to control soil-based organisms, and the fact that *P. ficus* are found on grapevine roots. *Steinernema yirgalemense* was found to be more effective in controlling populations of *P. ficus* in sand column tests than was *H. zealandica*, with neither EPN species being adversely affected by exposure to imidacloprid (thus making them both potential candidates for an integrated pest management complex with imidacloprid). However, the study concerned only assessed the ability of EPNs to control *P. ficus* on roots, where the latter are only found during the coldest months, and in the lowest numbers. *Planococcus ficus* populations move upwards on grapevine trunks during the summer months, congregating on leaves and buds, and increasing in number as the temperatures increase, with the populations declining in winter (Berlinger, 1977; Walton, 2003). This would limit an EPN strategy to control *P. ficus* that was purely soil-based, and, therefore, foliar applications must be considered.

The main objective of the current study was to investigate the effect of the addition of two adjuvants, namely Zeba® [starch-g-poly (2-propenamide-co-2-propenoic acid) potassium salt, Tongaat Hulett Starch] and Nu-Film-P® (poly-1-P-menthene) on treatments containing *S. yirgalemense,* with regard to the control of populations of *P. ficus* on foliage under semi-field conditions. Each adjuvant (and combinations thereof) were assessed for their effects on EPN efficacy in foliar application, as well as for their ability to increase EPN deposition and survival on grapevine leaves. The effect of variable nematode concentrations in nematode-adjuvant treatments was also investigated.

MATERIALS AND METHODS

Source of nematodes

Steinernema yirgalemense used in the present study originated from samples collected locally, which were maintained and recycled at Stellenbosch University. IJs were cultured *in vivo* by means of inoculating larvae of the mealworm beetle *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) with IJs. Infected mealworms were kept at 25°C until IJ emergence, before being transferred to White (1927) traps. The IJs harvested from White traps were stored in vented flasks, kept at 14°C, according to the guidelines set out by Kaya & Stock (1997). The flasks were gently agitated once a week to improve aeration. The IJs to be used in experimentation were used within one week of emergence.

Source of insects

A laboratory culture of *P. ficus* was established to ensure the granting of reliable access to the females concerned. The culture, which originated at the Agricultural Research Council (ARC) Infruitec-Nietvoorbij in Stellenbosch, South Africa, was propagated on butternut squash in a Perspex cage under ambient conditions. The cage, which was vented with mesh netting to allow for the circulation of air, was otherwise kept sealed to prevent the escape of mealybug crawlers. A fresh butternut was added once every three weeks, to allow the individuals concerned to migrate from the older butternut, which was then removed once rot had begun to set in. The females were handled with a paintbrush and tweezers, with the individuals concerned being removed only if they were not currently feeding, as damage to its mouthparts can affect the survivability of the insect.

Adjuvant field trial

To compare the effects of two adjuvants on the ability of *S. yirgalemense* to infect and cause mortality in *P. ficus*, an experiment was conducted at the Welgevallen Experimental Farm in Stellenbosch, Western Cape Province, South Africa. After washing in water with 0.01% hypochlorite solution, the grapevine leaves were thoroughly rinsed and left to dry. The leaves were cut into pieces to fit Petri dishes with a diameter of 13 cm. Two adjuvants were used, Zeba® (88% starch-g-poly) (United

Phosporus Ltd) and Nu-Film-P® (poly-1-p-menthene) (Hygrotech Properties). Treatments were: water only, IJs + water, IJs + Zeba®, and IJs + Zeba® + Nu-Film-P®. Nematode suspensions were formulated at 4000 IJs/ml and Zeba® was added at a concentration of 0.03%, and Nu-Film-P® at 0.06%. The treatments were prepared 1h prior to the trial.

For each treatment, eight Petri dishes were prepared, each containing a washed grapevine leaf, to which eight female *P. ficus* were added, reaching a total of 64 mealybugs per treatment. Treatments were applied to the Petri dishes via a calibrated handheld sprayer, after which the leaves were removed and left for 3min to eliminate excess runoff. Each leaf was then placed in a fine mesh pocket and sealed, in order to contain the mealybugs. The pockets were hung in the vineyard using a randomised block design, with 10 pockets being randomly distributed between four rows of vines. Each pocket was hung on alternating vines, 150 cm from the soil, with the outer rows and the first three vines of each row excluded to avoid edge effects. Ambient temperature and humidity were monitored in the vineyard using iButtons® (Maxim Integrated) placed in their own, separate mesh pocket. After 24h, the pockets were retrieved, with the mealybugs being removed from the leaves, rinsed, placed in Petri dishes lined with moistened filter paper, and incubated at 25°C. Mealybug mortality was assessed for mortality at 48h post-application. The experiment was repeated on a different date, with a fresh batch of nematodes.

Concentration field trial

The effect of IJ concentration on the ability of *S. yirgalemense* to cause mortality to *P. ficus* when formulated with Zeba® and Nu-Film-P® was investigated. Mesh pockets, grapevine leaves and mealybugs were prepared as previously described for the adjuvant field trial. The treatments applied included formulations of *S. yirgalemense* at concentrations of 1000, 2000 and 3000 IJs/ml, compared to a control treatment of water only. Each treatment (including the control) was formulated with 0.03% Zeba® and 0.06% Nu-Film-P®. After preparation, the leaves were placed in mesh pockets, hung in the vineyard, and assessed after 48 h. The temperature and humidity were monitored, using

temperature and humidity data loggers. The experiment was repeated on a different date, with a fresh batch of nematodes.

Morning and afternoon outdoor applications

The effects of adjuvants on nematode desiccation under field conditions were assessed. A grapevine at Welgevallen Experimental Farm was pre-wet, using a backpack sprayer of water and a suspension containing *S. yirgalemense* at a concentration of 2000 IJs/ml, Zeba® (0.03%) and Nu-Film-P® (0.06%). The application to the leaves was made using a calibrated handheld sprayer, with 3min being left to eliminate excess runoff. At 0, 30, 60, 120 and 240min post-application, three leaves were removed from the plant and two 2-cm^2 discs were cut from each leaf, for a total of six discs per time interval. Each disc was rinsed with 5 ml tap water, whereupon the number of living and dead nematodes was recorded. The application, which was done at 8:00 in the morning, was repeated at 14:00 in the afternoon. Nematodes that did not respond to either light or prodding were recorded as being dead. The experiment was repeated at a later date, with a fresh batch of nematodes.

Data analysis

The analysis of data obtained from all the trials was conducted on STATISTICA statistical analysis software version 13 (TIBCO Inc., 2017). Data from the adjuvant and concentration field trials were analysed using the ANOVA, while data from the outdoor deposition trial were analysed using generalised nonlinear models (GLZs), using a Poisson distribution and a log link function. For each experiment, the data from both trial dates were compared by means of an ANOVA to confirm the significant differences. Kruskall-Wallis tests were performed to confirm the results of the ANOVA and GLZ analyses. Bonferroni's test was applied for the post-hoc comparison of means. All significant differences were calculated to 95% probability level.

RESULTS

Adjuvant field trial

The mean temperature at EPN application was 19.4°C, with a minimum of 13.6°C and a maximum of 31.7°C, during the exposure period. The average temperature over the exposure period was 21.8°C. The relative humidity (RH) was recorded as being 69.5% at EPN application, ranging between 32.9 and 94.8% over the duration of the trial, with an average of 67.5% over the exposure period.

No significant difference was found between the main effects of treatment and time, allowing data from the two trials to be pooled. The percentage mortality of *P. ficus* post-exposure to each of the *S. yirgalemense*-containing treatments was analysed using a one-way ANOVA, with treatment as a factor. Analysis showed a significant difference in mortality between each treatment ($F_{(3,120)}$ = 144.94, p = <0.01), with each nematode treatment giving significantly higher mortality than the control (5.5% ± 2%) after 48h. Both adjuvant-based IJ treatments gave significantly higher mortality than did the IJs alone (28.1% ± 2%), with Zeba® + Nu-Film-P® being the most effective overall treatment (66.4% ± 4%), followed by Zeba® alone (43.0% ± 3%) (Fig. 4.1).

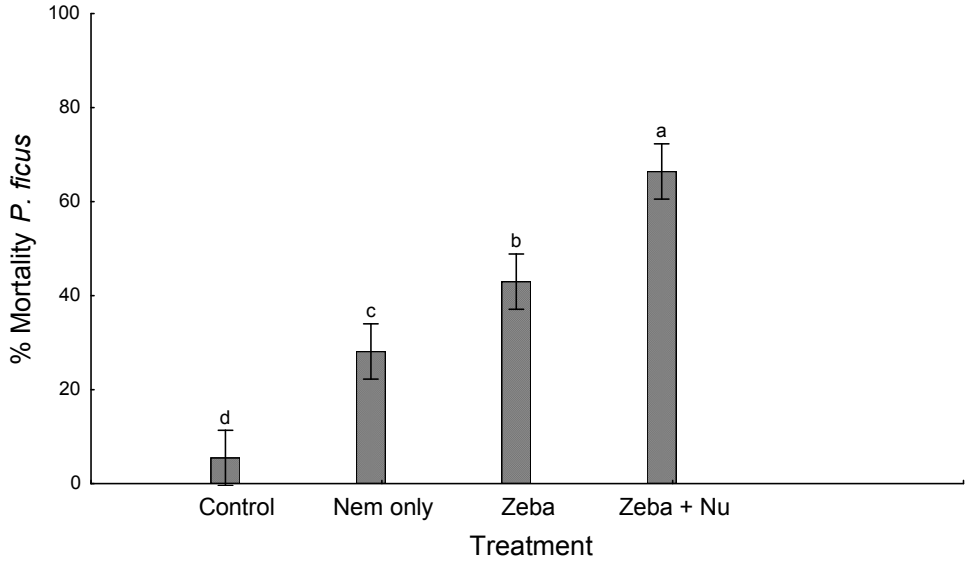

Figure 4.1. Mean percentage (95% confidence interval) mortality of *Planococcus ficus* on grapevine leaves, treated with 4000 IJs/ml *Steinernema yirgalemense*. Leaves were exposed in mesh pockets in a vineyard for 24h. Mortality was assessed 48h total post-application (one-way ANOVA: $F_{(3,120)}$ = 144.94, p = <0.01). Means of bars labelled with the same letter are not significantly different from one another.

Concentration field trial

The average temperature at EPN application (08:00) was 20.9°C, with the RH at 65.3%. Temperatures during the trial period ranged between 13.6°C and 31.5°C, with a mean of 21.5°C over the 48h

exposure period, and with the RH ranging between 32.1% and 94.8%, with a mean of 67.9% over the exposure period (Fig. 4.2).

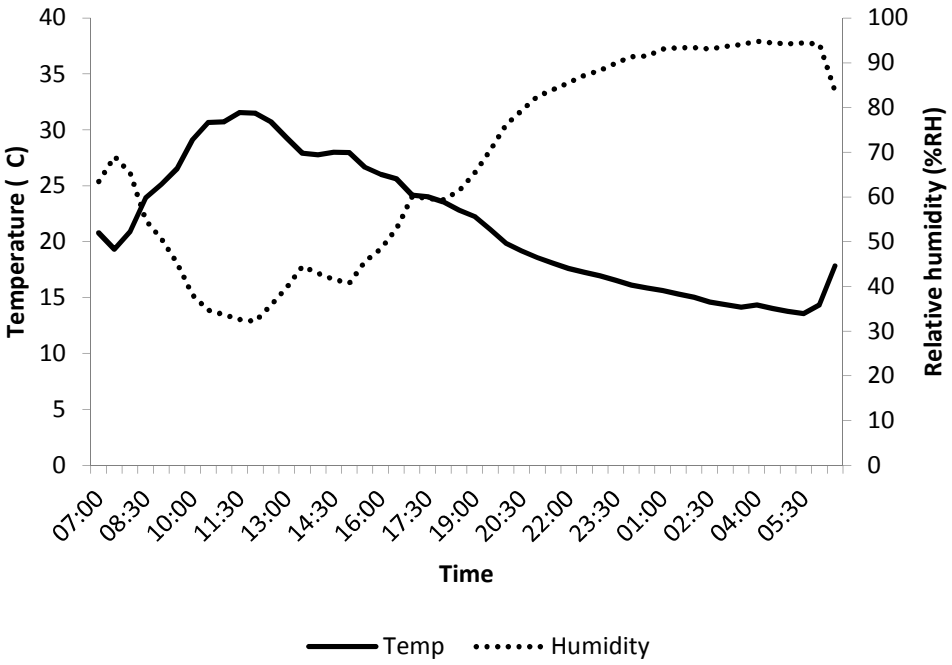

Figure 4.2. Climatic data recorded over the first 24h duration of the concentration trial.

The two field trials were analysed, with treatment and date as the main effects. As no significant differences were found between the two experiments, the resulting data were pooled. A one-way ANOVA was used to compare the effect on mealybug mortality of three different concentrations of *S. yirgalemense* with Zeba® + Nu-Film-P®. Each treatment was significantly different to the others ($F_{(3,112)} = 46.467$, $p = <0.01$), with the treatment with the highest concentration of 3000 IJs/ml being the most effective (43.8% ± 4%) after 48h, followed by the treatments with concentrations of 2000 IJs/ml (32.0% ± 3%) and 1000 IJs/ml (20.3% ± 4%), compared with the control (7.8% ± 3%) (Fig. 4.3).

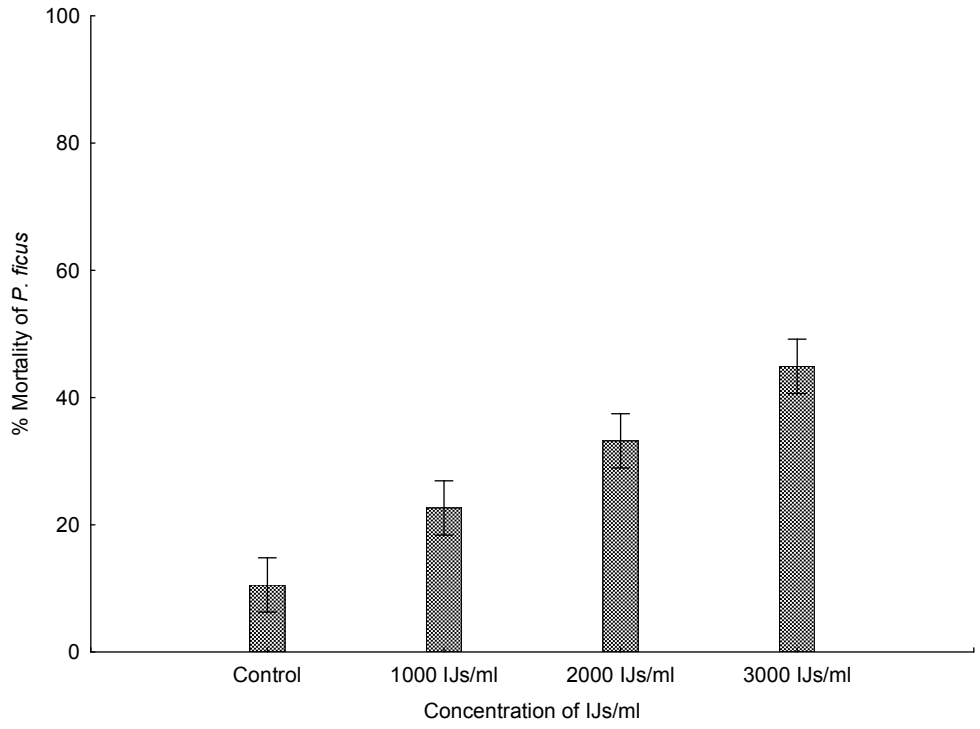

Figure 4.3. Mean percentage (95% confidence interval) mortality of female *Planococcus ficus*, using three different concentrations (1000, 2000 and 3000 IJs per ml) of *Steinernema yirgalemense*. Mortality was assessed 48h post-application (one-way ANOVA: $F_{3, 112} = 46.467$, $p = <0.01$). Means of bars sharing a letter are not significantly different from one another.

Morning and afternoon application

For the morning trial, temperature and humidity at the start of the trial (8:00) were 14.6°C and 93.2%, respectively. Temperatures ranged between 15.0 and 34.9°C during the exposure period, with an average temperature of 25.2°C. The RH ranged from 34.0 to 93.7%, with an average 60.2% during the trial period (Fig. 4.4). Conditions differed in the afternoon trial, with the temperature and RH, at the time of application (14:00), being 31.0°C and 39.9%, respectively. Temperatures during the 4-h period ranged between 20.4 and 31.0°C, with an average of 26.8°C. The RH ranged between 40.6 and 64.6%, with an average of 46.8% over the period concerned (Fig. 4.5).

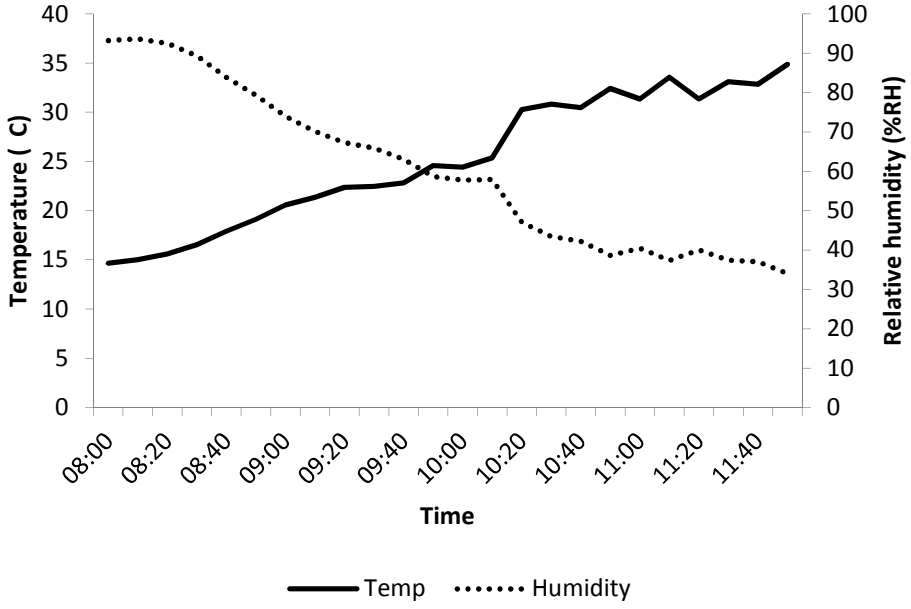

Figure 4.4. Climatic data recorded over the 4h exposure time of the morning outdoor deposition trial.

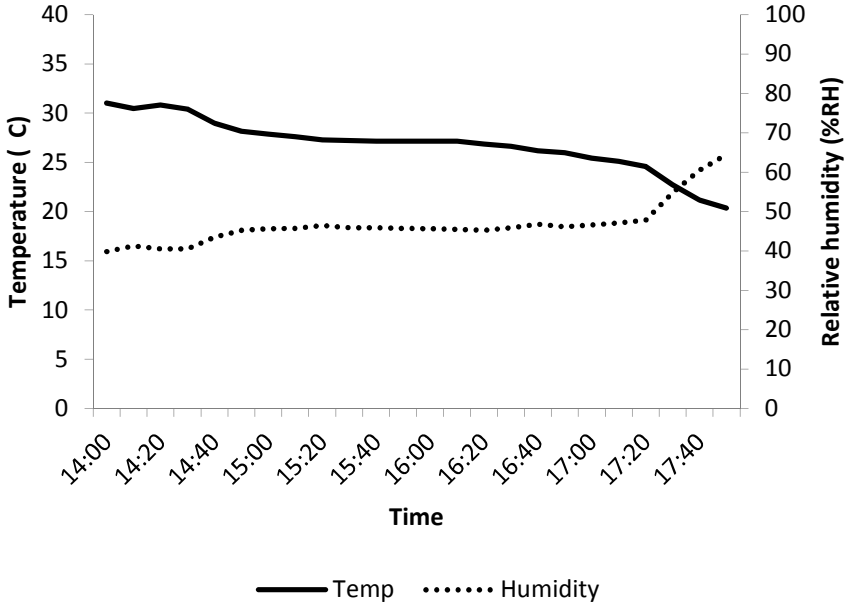

Figure 4.5. Climatic data recorded over the 4 h exposure time of the afternoon outdoor deposition trial.

Generalised nonlinear model analysis (GLZ) was used to compare the counts of live nematodes collected from the treated leaves. Overall, the number of live *S. yirgalemense* retrieved from leaf discs differed significantly between 8:00 and 14:00 ($p < 0.01$), and by time interval post-application ($p < 0.01$) (Fig. 4.6). In the morning application, 4.7 nematodes were recovered after 4h, in

comparison with the 6.1 nematodes that were recovered immediately after application. In the afternoon application, 4.5 nematodes were retrieved directly after application, in comparison to the 0.5 nematodes retrieved 4h later.

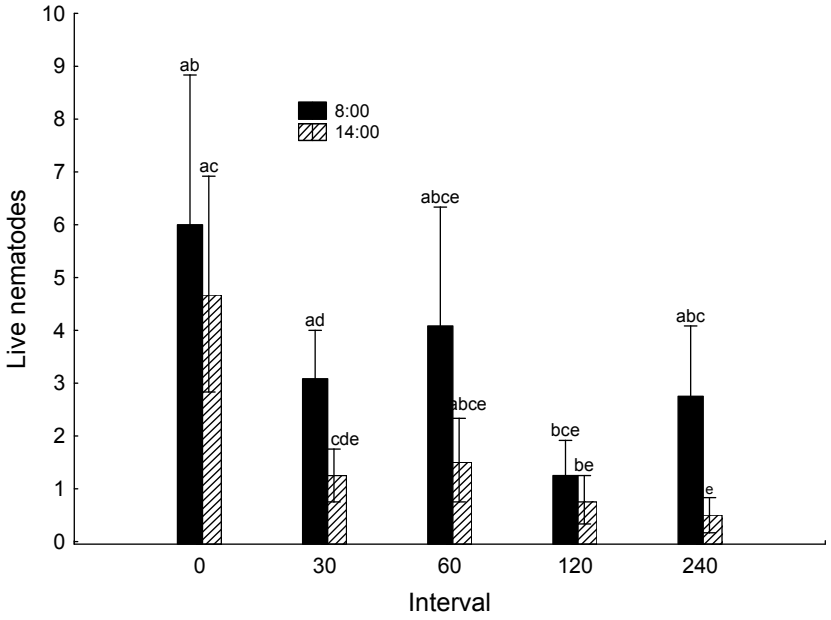

Figure 4.6. The mean number of nematodes collected from leaf discs at timed intervals post the application of a suspension of *Steinernema yirgalemense*, Zeba® and Nu-Film-P®. Nematodes were applied to leaves using a handheld sprayer, at a concentration of 2000 IJs/ml. The number of live nematodes present at each time interval was compared (Wald X^2 (4) = 13.239, p = 0.017). Means of bars sharing a letter are not significantly different from one another.

DISCUSSION

The search for environmentally friendly biocides to control insect pests has led to the investigation of EPNs for use against a wide variety of target pests. EPNs are highly pathogenic to insects, with the wide variety of species being available allowing for selection for optimal use against specific pest insect life stages. Laboratory bioassays inoculating foliage-based insect pests with EPNs have shown high rates of mortality, making EPNs a promising candidate for the control of such pests. However, foliage-based application of EPNs have generally failed to live up to the potential of soil-based EPN treatments, largely due to the maladaptation of EPN species to the above-ground environment. The addition of adjuvants has been investigated in efforts to combat the negative effects of above-ground environmental conditions, including the exposure to temperature, RH and UV radiation outside of the tolerance ranges of the specific EPN species used. This is a field with the potential for near-limitless

study, as new adjuvants are constantly being developed, and new EPN species are constantly being described, each with their own properties and context-specific interactions. In the current study, two adjuvants (Zeba® and Nu-Film-P®) were compared in terms of their effects on the efficacy of *S. yirgalemense*, an indigenous South African EPN species, when applied to control *P. ficus* on grapevine foliage.

The efficacy of Zeba® in improving EPN activity in above-ground environments had previously been demonstrated by De Waal *et al.* (2013), in terms of which Zeba® was added to suspensions containing *H. zealandica* for the control of codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae), on the trunks of pear trees in a South African orchard. The addition of Zeba® to EPN suspensions was found to improve both the survival and the infectivity of *C. pomonella* by *H. zealandica*, compared with water alone. Additionally, Van Niekerk (2012) demonstrated that prolonged exposure to Zeba® (24h) had no significant negative effect on the survival of *S. yirgalemense*. Zeba® was, therefore, selected as a promising candidate for the improvement of *S. yirgalemense* performance on grapevine foliage.

Nu-Film-P® (poly-1-P-menthene) is used as a spreader and sticker adjuvant for the application of chemical pesticides in South African vineyards, having been demonstrated to improve the deposition of fenhexamid, a fungicide, on Chardonnay grapevine foliage significantly (Van Zyl *et al*., 2010). Van Niekerk (2012), on assessing the survival of *S. yirgalemense* in suspension with Nu-Film-P® over time, determined that no significant increase in the mortality of *S. yirgalemense* occurred when it was formulated with Nu-Film-P® for up to 6h, and no significant decrease occurred in the mortality of *P. citri* post-storage. In the current study, the effect of Zeba®, as well as the combination of Zeba® and Nu-Film-P®, on the ability of *S. yirgalemense* to infect and cause mortality in female *P. ficus* was assessed. An EPN treatment under field conditions, containing a combination of Zeba® and Nu-Film-P®, was found to be the most effective treatment overall, achieving 66% mortality of the female *P. ficus* after 48h. The aforementioned mortality was significantly higher than that which was attained with treatments containing either adjuvant alone. Zeba® alone was the next

most effective treatment, achieving 43% mealybug mortality after 48h. The results demonstrate that the addition of Zeba® and Nu-Film-P® to *S. yirgalemense* treatments has a positive effect on the control of *P. ficus* on foliage. The benefits of each adjuvant appear to be additive. The Nu-Film-P® alone treatment increased *P. ficus* mortality by 22% compared to the control, with the treatment containing Nu-Film-P® and Zeba® giving a slightly higher mortality than did Zeba® alone. The finding is in contrast to that of Van Niekerk (2012), who assessed the ability of *S. yirgalemense* and *H. zealandica* to control the citrus mealybug, *P. citri*, combined with both Zeba® and Nu-Film-P® applied to infested citrus leaves. A growth chamber bioassay showed that all nematode-containing treatments improved *P. citri* mortality, but that the combination of Zeba® and Nu-Film-P® was the only treatment to offer significantly higher mortality of *P. citri* than did the nematodes alone. The combination of Zeba® and Nu-Film-P® offered no significant increase in mortality over the use of Zeba® alone. However, in the current field trials, only nematode treatments containing Zeba® were able to obtain significantly higher mortality, with no significant difference being observed between treatments containing Zeba® alone, compared with treatments containing both Zeba® and Nu-Film-P®. The difference in results attained may be ascribed to the different structures of the leaves used, with citrus leaves being firmer and more waxy than are grapevine leaves, on average. Nu-Film-P® is a spreader and sticker, and, as such, might have been more effective on grapevine leaves, whose surface is less hydrophobic.

Future research in this area should investigate the ability of Zeba® and Nu-Film-P® to improve *P. ficus* mortality, when applied with *S. yirgalemense* under true field conditions. As the current study used a semi-field approximation of application techniques, its conclusions are not directly applicable using traditional pesticide application methods, which typically use large spray machinery, blanket spraying, and different applicators. Additionally, in the present study, mealybugs were sprayed directly, and placed inside mesh pockets. However, as was covered in Chapter 1, a key concern of pesticide applications against the vine mealybug is their tendency to occupy cryptic habitats, thus shielding them from pesticidal application. A future study should investigate the ability of EPNs to

infect female mealybugs by means of actively moving into cryptic habitats where the insects reside, which is also a more conducive microhabitat for the nematodes themselves, thus offering a significant potential advantage over the use of chemical pesticides.

In the current study, an experiment was carried out to determine the effects of varying *S. yirgalemense* concentration on the mortality of female *P. ficus*. Each of the three EPN concentrations used (1000, 2000 and 3000 IJs/ml) resulted in significantly higher mortality after 48h. Additionally, *P. ficus* mortality increased linearly with higher nematode concentration, with each concentration differing significantly from the last. *Planococcus ficus* mortality at 1000 IJs/ml differed significantly from the control, with the mortality at 2000 IJs/ml being 32%, and with it being 44% at 3000 IJs/ml. The above suggests that EPN concentration can be increased for predictable increases in *P. ficus* mortality under such conditions. The suggestion is in keeping with the research that has been conducted by Le Vieux and Malan (2013b), who assessed the effect of increasing the concentration of three EPN species on individual *P. ficus* mortality. A similar increase in mortality was also observed as the EPN concentration was increased from 0 to 80 IJs per mealybug. This is comparable to the increase in mortality observed per 1000 IJs/ml in Figure 4.3. In contrast, De Waal (2008) examined the effects of increasing concentrations of *H. zealandica* applications on the mortality of diapausing codling moth, *C. pomonella*, larvae, with EPN concentrations ranging from 80 to 640 IJs/ml. Despite a positive relationship being found between increasing concentration and codling moth mortality, no significant difference was observed between the mortality caused at 80 to 160 IJs/ml, and the mortality caused at 640 IJs/ml. An LD_{90} of 275 IJs/ml was established, implying diminishing returns for increasing concentrations after such a point. Future research should investigate the upper limit, if any, of increasing concentrations of *S. yirgalemense* on *P. ficus* mortality, when applied with Zeba® and Nu-Film-P®.

Additionally, the effect on EPN survival on foliage caused by the climatic differences observed in morning and afternoon applications was assessed. The mean temperature and humidity over the

experimental period varied greatly, with the temperature at 14:00 being 16°C higher than at 8:00. The RH was also much lower at 14:00 (40%), compared with at 8:00 (93%). Overall, the foliar survival of EPNs was significantly lower when they were applied in the afternoon. Additionally, the number of living nematodes recovered from leaf discs in the afternoon was lower at all intervals, compared with the same intervals when applied in the morning trial. De Waal *et al*. (2017) observed similar findings with respect to the interaction between *H. zealandica* and *C. pomonella*. The researchers concerned attached cardboard strips containing diapausing codling moth larvae to trees in orchards, to which they applied nematodes just after sunrise on the day of the trial. The setup was repeated by applying the nematodes at sunset. Mortality of 80 to 100% was recorded when the nematodes were applied to codling moth larvae at sunrise, compared with <50% mortality when the nematodes were applied at sunset. In general, morning application appears to be superior to evening application with regard to EPN survival and infectivity.

The above-mentioned results illustrate the importance of a proper timeframe selection for application, as adjuvants alone are insufficient to counter the effects of climatic conditions completely. In order to be effective, knowledge of the local climatic conditions, as well as of the temperature/humidity niche breadth of the EPN species used, is essential. In the case of *S. yirgalemense*, with the weather conditions at 8:00 being closer to the ideal for application than they were at 14:00 served to establish that 100% RH and temperatures of around 25°C (Chapter 2) seemed to be ideal for the EPN infection of female *P. ficus*.

Future research would be useful in determining the relationship between temperature and humidity. Applications in the case of the current study took place in March 2017, and, over the 24h period assessed, the temperature and humidity conditions did not align ideally – the temperatures at maximum humidity were lower than the ideal, and the humidity at optimal temperatures (Chapter 2) was also low. It would, therefore, be of interest to investigate the relationship between temperature and humidity to determine the most important factor in terms of EPN success on foliage. Additionally, the effects of irrigation on the foliar application of EPNs in the control of *P. ficus* should be

investigated. EPN survival on foliage can be improved when applications occur post-rainfall (Mráček, 2002). However, rain forecasting in South Africa is less reliable than it is elsewhere, such as in Europe. Downing (1994) demonstrated the potential of pre- and post-application irrigation when *H. bacteriophora* was applied in the control of two Coleopteran species on Kentucky bluegrass, achieving consistent pest mortality (>80%), compared to unirrigated controls. The finding in question is in keeping with the conclusions of Odendaal *et al.* (2016), who investigated the relative importance of temperature and humidity on three EPN species when they were applied to control codling moth in South African apple orchards. While certain of the EPN species tested occupied different temperature niches, being found to perform best within those niches, the increase in RH was, overall, found to be the most effective factor in the improving of EPN control over codling moth. *Steinernema feltiae*, a more cold-adapted European EPN species, was also found to perform better than did *S. yirgalemense*, which originates in sub-tropical Mpumalanga, when applied in late autumn / early winter. Therefore, it would appear that EPN species should be selected for the expected temperature niche during which they will be applied, and application techniques should focus instead on maintaining the appropriate humidity levels within the application area for as long as possible.

One possible area of grape production that might synergise with EPN applications is the use of table grape vineyards covered with shade netting. Increasing global temperatures tend to lead to negative effects on wine grapes grown in hot regions. For example, Sémillon grapes demonstrate a decrease in the sugar content of grapes and photosynthesis when exposed to 40°C temperatures (Greer & Weston, 2010). Artificial shading methods are commonly employed in table grape vineyards to manage the prevailing temperature, after studies have been carried out to assess the impact of shading on wine grape vineyards. Cartechini & Palliotti (1995), on assessing the effects of three levels of cover (100%, 60% and 30% sunlight penetration) on the temperature and humidity in a Sangiovese vineyard, found that the temperature decreased and the humidity increased in covered vineyards, with the water vapour pressure deficit (VPD) declining from 1.43, in uncovered vineyards, to 1.28, in vineyards with 30% sunlight penetration. Similar results have been demonstrated with regards to

Shiraz (Caravia *et al*., 2016) and Sémillon grapes (Greer & Weston, 2010). Besides their intended purpose in ameliorating conditions for wine grape development, artificial shading might also serve to ameliorate conditions for EPN activity by means of lowering temperatures and by means of (critically) causing relative humidity levels to increase.

Overall, the ability of an adjuvant-based *S. yirgalemense* treatment to obtain high mortality of female *P. ficus*, under semi-field conditions, is promising in terms of the development of a potential foliar biopesticide containing *S. yirgalemense*. Noteworthily, however, the results concerned were obtained from the direct spraying of mealybugs, with work remaining to be done on developing an effective means of application for mealybug colonies living in cryptic habitats on grapevines. Nevertheless, the current study demonstrates that it is possible for high concentrations of *S. yirgalemense* to obtain >65% mortality in female mealybugs on grapevine foliage, when their action is aided by adjuvants and applied in windows of time during which optimal climate conditions are present. Research into techniques for maintaining optimal environmental conditions, for both grape and nematode, is the next step to be undertaken in the search for an effective nematode-based solution to the existing problems in this field.

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CHAPTER 5

CONCLUSION

The overall aim of this study was to contribute to the development of methods aimed at employing entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae and Steinernematidae) as a biocontrol agent against the vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), on grapevine foliage. The objectives of this study were firstly, to identify a suitable EPN candidate through screening several endemic species for efficacy in the control of *P. ficus* in the laboratory, along with optimal environmental conditions for their activity. Secondly, the effects of adjuvants were assessed on the mortality of *P. ficus*, as well as the deposition of nematodes onto grapevine leaves, when added to nematode formulations. Thirdly, EPN and adjuvant combinations were assessed for their ability to cause mortality in *P. ficus* in semi-controlled environments, such as in a growth chamber and in the glasshouse. Lastly, these combinations were then assessed for *P. ficus* mortality and deposition in field conditions, in a vineyard.

The first objective was investigated under laboratory conditions. *Steinernema yirgalemense* had previously been demonstrated to be the optimal species for control of the vine mealybug by applying several previously-described EPN species. Further screening was thus required in order to compare the activity of *S. yirgalemense* to three newly-described endemic species (*Steinernema jeffreyense*, *Heterorhabditis noenieputensis*, and unnamed *Steinernema* spp. isolate WS9). Of the three species, *H. noenieputensis* demonstrated the highest level of control during screening, with both it and *S. yirgalemense* attaining > 60% control of P. ficus in laboratory bioassays. Successful infection was confirmed when *S. yirgalemense* and *H. noenieputensis* individuals were recorded in the cadavers of P. ficus post-application. *Steinernema yirgalemense* was selected as the EPN candidate to use going forward, due to difficulties in the culture of heterorhabditids. However, *H. noenieputensis* will be a very promising candidate for future study, as methods of mass-culturing heterorhabditid species improve.

The effects of different lengths of exposure of *P. ficus* females to *S. yirgalemense* were then assessed. *Planococcus ficus* mortality increased with increasing time intervals exposed to *S. yirgalemense* up to 3 h, after which no significant increase in mortality was observed. These results suggest that this is the minimum amount of time for which EPNs must be allowed to survive on exposed foliage in order to obtain maximum efficacy. Further laboratory bioassays investigating temperature and humidity, confirmed that *S. yirgalemense* causes the highest mortality in temperatures of 25°C to 30°C, and at as close to 100% relative humidity (RH) as possible.

In order to fulfil the second objective, two adjuvants viz. Zeba® and Nu-Film-P®, were assessed for their effects on *S. yirgalemense* suspensions with regard to EPN deposition on grapevine leaves. Results showed that spray application of the two adjuvants depositing significantly greater numbers of nematodes onto leaves, than either Nu-Film-P® alone or the control, though not more than Zeba® alone. These formulations were then assessed for efficacy in causing *P. ficus* mortality in a growth chamber environment, in order to fulfil the third objective. The application of both Zeba® and Nu-Film-P® caused significantly higher mortality than all other treatments, in both the growth chamber and the glasshouse. No reduction in efficacy was observed from moving this treatment from a highly-controlled environment (the growth chamber) to a semi-controlled environment (the glasshouse).

The ability of these adjuvants, combined with nematode suspensions to promote EPN survival on foliage, and improve *P. ficus* mortality on grapevines in vineyards, was assessed using a semi-field trials in order to fulfil the final objective. Though mortality was lower overall than the indoor trials, the combination of *S. yirgalemense,* Zeba® and Nu-Film-P® achieved significantly higher mortality of *P. ficus* than any other treatment, with > 60% mortality observed after 48 hours. Reducing EPN concentration in this treatment caused a proportionate decrease in EPN mortality. In order to assess EPN viability on foliage over time at different times of day, EPNs were applied to grapevine leaves with Zeba® and Nu-Film-P® in the morning (conditions of high humidity/low temperature) and in the afternoon (high temperature/low humidity). More live EPNs were found to be present on

grapevine leaves at each time interval after morning application, when compared to afternoon application, despite the fact that temperatures in the afternoon were close to the ideal temperatures for *S. yirgalemense*. This might indicate that optimal humidity, rather than optimal temperature, is the critical factor for EPN success on foliage.

The main limiting factor in the application of EPNs on foliage, and other above-ground environments, is the susceptibility of EPNs to environmental and climatic conditions, such as extremes of temperature and insufficient humidity. The results of this study indicate that these limits can be partially overcome in order to formulate EPN-based treatments, which can achieve > 60% mortality of *P. ficus* on grapevine foliage. The critical factor appears to be humidity, which must be maximised. Anti-desiccant adjuvants, and application timed to coincide with maximum daily levels of relative humidity, were used in this study to achieve the highest relative humidity possible. Other means, such as overhead irrigation of vineyards and the use of shade netting, should be investigated for their ability to improve the activity of *S. yirgalemense* on grapevine.

The next logical step from this study is to apply these treatments to vineyards infested with *P. ficus*. The semi-field trial, as performed, applied EPN treatments to *P. ficus* individuals on leaf discs, which were hung in the vineyard to approximate field conditions. Though this approach demonstrated the effects of above-ground conditions on EPN treatments, it does not accurately replicate the behaviour of *P. ficus* on grapevines. One of the key challenges to the application of chemical insecticides in the control of *P. ficus* arises in the chosen habitats of the mealybugs themselves, which are often sheltered and cryptic, located beneath raised bark on grapevine trunks or beneath several layers of leaves. These cryptic habitats are also an optimum micro habitats for EPNs, as it is away from UV-light, with high humidity and the preferred habitat of the target insect. One of the potential key benefits of EPNs as a control agent of *P. ficus* is that, as living creatures, EPNs may be able to seek out and infect *P. ficus* individuals in habitats where they are impervious to chemical pesticides. The semi-field trial in this study, demonstrates the ability of *S. yirgalemense* to cause mortality in *P. ficus* when directly applied in the field in exposed conditions, while the deposition field trial

demonstrated that EPNs could persist on leaves for as long as 4 h. Trellised table grapes destined for export, lend to an ideal environment for EPNs. Future work should investigate the ability of EPNs to seek out *P. ficus* females in natural infestations on grapevines, and record subsequent infection and mortality, if they are ever to function in a manner similar to chemical insecticides.

One potential area of further study would be to investigate application technology. Typically, attempts to "weaponise" EPNs for use against insect pests of foliage have tended towards developing methods, which use existing pesticide spray equipment for the purpose. The reasoning behind this is that EPN treatments are more attractive to end users if they do not require significant buy-in of new equipment. Current practices and equipment for spray application, and the differences therein, should be investigated for their effects on the efficacy of *S. yirgalemense* with regard to causing *P. ficus* mortality in natural infestations in vineyards.

This study demonstrates the promise that *S. yirgalemense* has shown, in being an effective biocontrol agent of *P. ficus* on grapevine foliage, and demonstrates that such control is possible. Even though, in the worst-case scenario, a 60% control in field application is envisaged, the additional benefits to the application of EPNs against mealybugs are not accounted for. These benefits include the positive effect on the environment, the removal of individuals mealybugs with chemical resistance from population, application close to harvest of grapes destined for export, as there will be no problems with chemical residues, indirect control of other grapevine pests such as fruit fly, weevils and false codling moth. The results from this study, combined with the additional benefits, strengthen the next step in the process to refine these treatments and application methods into a viable treatment, which can be proven for use in commercial vineyards, as part of an integrated pest management system for the control of the vine mealybug.